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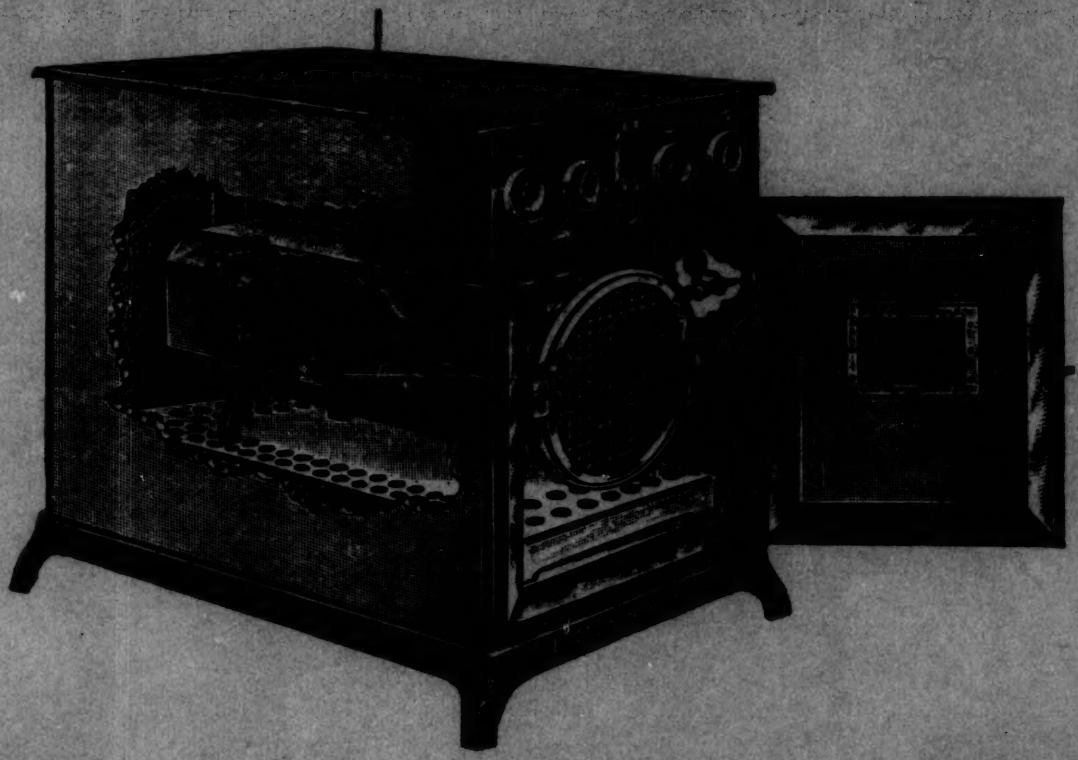

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

TENTH ANNUAL MEETING.

Boston, Mass., December 27–29, 1915.

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

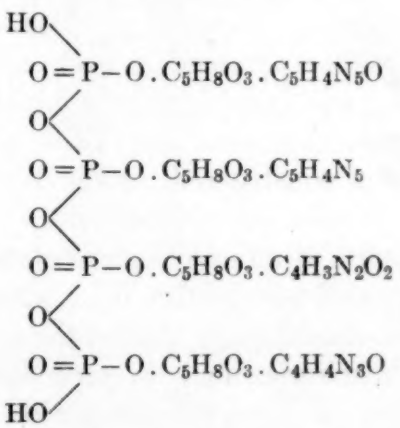
PRESIDENTIAL ADDRESS.

AN INDIRECT METHOD OF DETERMINING PYRIMIDINE GROUPS IN NUCLEOTIDES.

By WALTER JONES.

When the question of a presidential address was recently brought to my attention, I chose the title which appears in the program ("Methods in Biochemistry") and prepared to deal with the development of biochemistry in its broader aspects and with the vital rôle that methods have played in this development. But a number of considerations, especially the great length of the program, have induced me to abandon my original intention and confine the discussion to a particular chemical method which I have recently studied in collaboration with Miss Catherine Riley of Washington.

Questions pertaining to the chemistry of nucleic acid can be conveniently referred to the following formula:



This formula represents nucleic acid as a tetra-nucleotide made up of four mono-nucleotides united to one another by their phosphoric acid groups, and it indicates the paths along which the substance may be expected to undergo decomposition whether

by chemical means or under the influence of physiological agents. Such a decomposition could produce a variety of substances: tri-nucleotides, di-nucleotides, mono-nucleotides, nucleosides, pyrimidine derivatives, purine derivatives, pentose, and phosphoric acid. So that, while the hydrolytic decomposition of nucleic acid is not a difficult matter to discuss, its experimental investigation is almost impossible; for of the decomposition products enumerated, only one (phosphoric acid) admits of exact quantitative determination. The chief difficulty lies in the estimation of the pyrimidine derivatives. But since the *purine derivatives* can be estimated with a fair degree of accuracy, the principal obstruction to investigation in this field would be removed if one could devise a precise experimental method of determining the ratio of the number of combined purine groups to the number of combined pyrimidine groups. From this point of view the following experiments are of interest.

Ten portions of commercial yeast nucleic acid from the same specimen were weighed into appropriate vessels and in one of these the total phosphoric acid was determined. The other nine portions were submitted to hydrolysis with 5 per cent sulfuric acid at 100° for various periods from $\frac{1}{4}$ hour to $7\frac{1}{2}$ hours and after removal of the guanine with ammonia, the phosphoric acid was precipitated with magnesia mixture and weighed as magnesium ammonium phosphate.[1] The weight of magnesium ammonium phosphate obtained in each experiment was divided by the weight of nucleic acid from which it was produced and from the comparable results so obtained (Table I, Column 4) the upper curve in Fig. 1 was constructed. (The method of construction is so obvious as to require no explanation.)

This curve admits of an easy interpretation. Its rapid initial rise, sharp turn, and termination in a straight line slightly inclined to the axis, show clearly that in the acid hydrolysis of yeast nucleic acid, phosphoric acid is progressively liberated from two sources. From the one, the production is rapid and complete in less than 2 hours; from the other, it is much slower, continues beyond the time consumed in these experiments, and is so regular that its amount is definitely seen to be equivalent to 10 mg. of

[1] The admissibility of magnesium ammonium phosphate as a form for weighing phosphoric acid will be taken up in a special contribution.

TABLE I.

Hydrolysis of Commercial Yeast Nucleic Acid with 5 Per Cent Sulfuric Acid 20 Cc. per Gm.

Nucleic acid.	Duration of hydrolysis.	Magnesium ammonium phosphate $MgNH_4PO_4.6H_2O$.			
		Obtained.	Per gm. of nucleic acid.	Pyrimidine correction.	From purine nucleotides.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
1.1782	¼	0.1060	0.090	0.003	0.087
0.7347	½	0.1284	0.175	0.005	0.170
0.8644	1	0.2315	0.269	0.010	0.259
0.9837	2	0.3125	0.318	0.020	0.298
0.9251	3	0.3038	0.329	0.030	0.299
1.0305	4	0.3488	0.338	0.040	0.298
0.8333	5	0.2876	0.345	0.050	0.295
0.9927	6	0.3565	0.359	0.060	0.299
0.9667	7½	0.3625	0.375	0.075	0.300
0.8179	Total.....0.5112		0.590*	Half.....0.295	

* See note at end of article.

magnesium ammonium phosphate per hour. If we subtract this amount from the figures of Column 4, Table I, we obtain the figures of Column 6, from which the lower curve of Fig. 1 has been constructed. This curve ends in a straight line, parallel to the axis, whose ordinate corresponds to half the total phosphoric acid obtainable from nucleic acid; or in other words, the

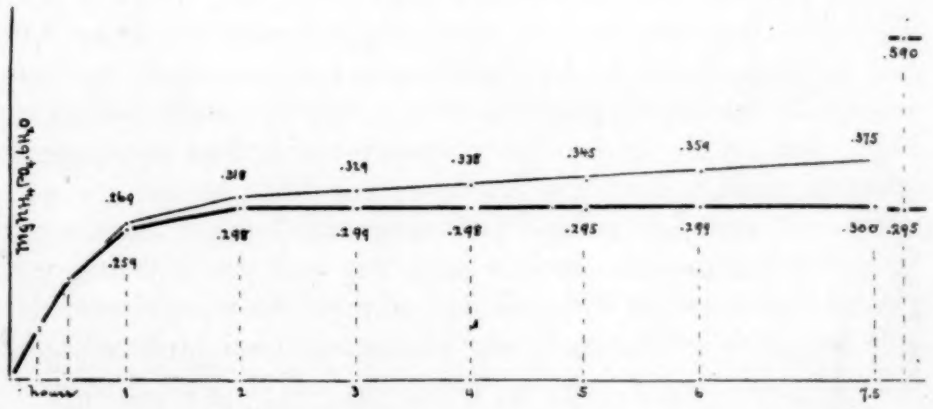


FIG. 1.

phosphoric acid which is rapidly and completely liberated by acid hydrolysis of yeast nucleic acid is half the total.

If time permitted it could easily be shown that at the point where the curve makes its sharp turn, the purines have been completely set free while the pyrimidines are still combined. Moreover the existing literature forces the conclusion that the two sources of phosphoric acid which we are discussing are the *purine* nucleotides and the *pyrimidine* nucleotides.[2] As the two sources of phosphoric acid are equal in amount the purine groups of nucleic acid must be equal in number to the pyrimidine groups.

TABLE II.

Purified Nucleic Acid. First Fraction with Glacial Acetic Acid.

Nucleic acid.	Duration of hydrolysis.	Magnesium ammonium phosphate $MgNH_4PO_4.6H_2O$.			
		Obtained.	Per gm. of nucleic acid.	Pyrimidine correction.	From purine nucleotides.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
0.7519	1	0.1968	0.262	0.01	0.252
1.0308	2	0.3278	0.318	0.02	0.298
1.0024	3	0.3290	0.328	0.03	0.298
0.9703	4	0.3304	0.341	0.04	0.301
0.9009	5	0.3115	0.346	0.05	0.296
0.6846	Total.....0.4265		0.590*	Half.....0.295	

* See note at end of article.

This proposition has been accepted for a long time but rests only upon negative evidence, and its authorship would be difficult to discover if a third pyrimidine derivative were to be found among the hydrolytic products of nucleic acid. Such a discovery would now simply lead to the assumption of a third purine group in nucleic acid.

8 years ago Levene purified commercial yeast nucleic acid by dissolving the substance in ammonia and precipitating with glacial acetic acid.[3] This method of purification is of considerable importance because it was afterwards used by Levene and

[2] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1911, xliv, 1027.

[3] Levene, P. A., *Biochem. Z.*, 1909, xvii, 120.

his coworkers in their work on nucleotides, and it is naturally of interest to know how such a preparation of nucleic acid would conduct itself when submitted to the method of partial hydrolysis described. Commercial yeast nucleic acid was therefore purified by Levene's method but was *fractionally* precipitated with glacial acetic acid and in such a way that five preparations were obtained. The 1st, 4th, and 5th of these were examined and the results are shown in Tables II, III, and IV, and Figs. 2, 3, and 4.

As far as concerns the liberation of phosphoric acid by acid hydrolysis, these three preparations differ neither from one another nor from commercial nucleic acid. They contain different amounts

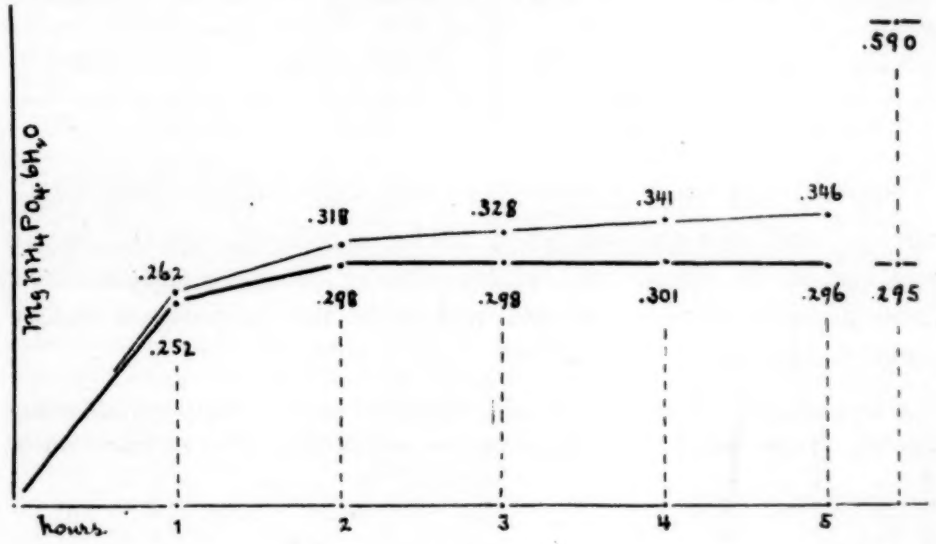


FIG. 2.

of moisture and therefore give different quantities of phosphoric acid, but each gives off accurately half of its phosphoric acid rapidly and the other half slowly; each contains an equal number of purine and pyrimidine groups.

To summarize: The purine-phosphoric acid of a nucleotide preparation may be determined by heating the preparation for 2 or more hours with 5 per cent sulfuric acid and subtracting from the estimated free phosphoric acid the equivalent of 10 mg. of magnesium ammonium phosphate per hour. The pyrimidine phosphoric acid is the difference between this value and the total phosphoric acid.

TABLE III.

Purified Nucleic Acid. Fourth Fraction with Glacial Acetic Acid.

Nucleic acid.	Duration of hydrolysis.	Magnesium ammonium phosphate $MgNH_4PO_4.6H_2O$.			
		Obtained.	Per gm. of nucleic acid.	Pyrimidine correction.	From purine nucleotides.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
1.1669	1	0.3097	0.266	0.01	0.256
0.9881	2	0.3334	0.338	0.02	0.318
0.8772	3	0.3087	0.352	0.03	0.322
0.8238	4	0.2966	0.360	0.04	0.320
0.6984	5	0.2578	0.369	0.05	0.319
0.8350	Total.....0.5448		0.632*	Half.....0.316	

* See note at end of article.

The method thus briefly described was studied with yeast nucleic acid but was expected to be principally useful in its application to other nucleotides. Some of these applications have already been made and will form the subjects of future communications.

The preparations of nucleic acid employed in this work contain magnesium. Its amount is small and varies considerably from preparation to

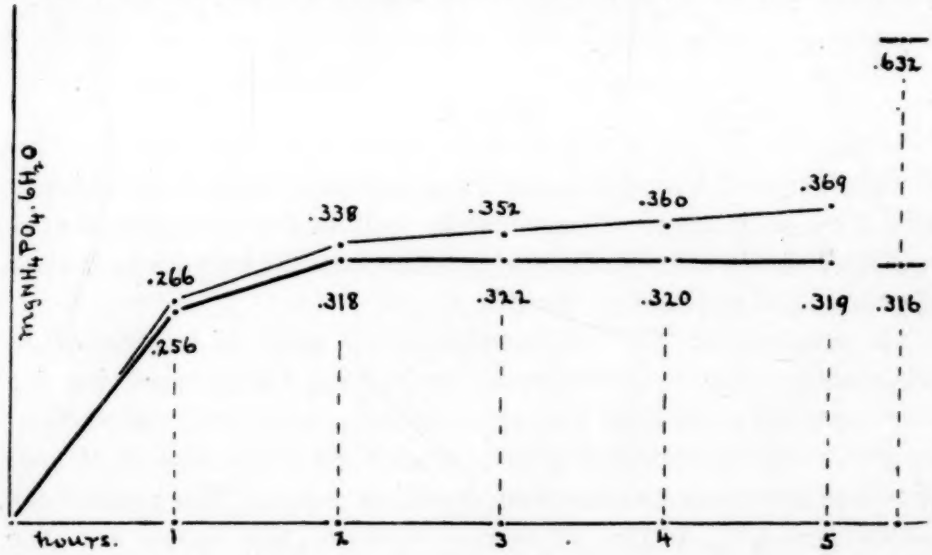


FIG. 3.

TABLE IV.

Purified Nucleic Acid. Fifth Fraction with Glacial Acetic Acid.

Nucleic acid.	Duration of hydrolysis.	Magnesium ammonium phosphate $MgNH_4PO_4.6H_2O$.			
		Obtained.	Per gm. of nucleic acid.	Pyrimidine correction.	From purine nucleotides.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
0.8805	1	0.2289	0.260	0.01	0.250
0.8434	2	0.2743	0.325	0.02	0.305
0.8532	3	0.2866	0.336	0.03	0.306
0.7346	4	0.2571	0.350	0.04	0.310
0.6624	5	0.2387	0.359	0.05	0.309
0.5307	Total.....	0.3274	0.612*	Half.....	0.306

* See note at end of article.

preparation so that it is not to be regarded as a constituent of the nucleic acid molecule. But its presence produces a slight disturbance in these experiments. When the products of hydrolysis are treated with ammonia for the removal of guanine some phosphoric acid is at the same time precipitated as magnesium ammonium phosphate and thus escapes estimation. In order to correct this error, the magnesium was determined in a large quantity of each preparation and its calculated equivalent of magnesium ammonium phosphate was subtracted from half that obtained in the total phosphoric acid determinations. The results are given in Table V.

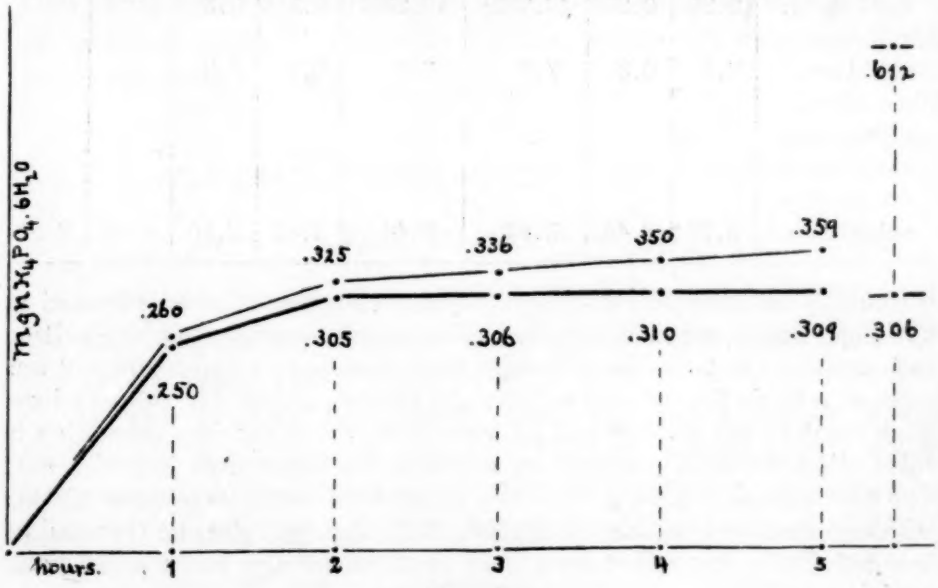


FIG. 4.

In this table have been included the results of moisture determinations and calculated total phosphorus percentages for the dry substances. The latter offer a curious suggestion.

It is not easy to determine the moisture of nucleic acid. A preparation can be brought to a fairly constant weight at any given temperature but will continue to lose weight when the temperature is raised. One is therefore somewhat at a loss to decide at what temperature the drying should be done. But in order to obtain as high phosphorus percentages as could

TABLE V.

The Phosphorus Percentages of Various Preparations of Nucleic Acid.

	Commercial yeast nucleic acid.	Nucleic acid fractioned with glacial acetic acid.					Levene's preparation.	Theoretical.
		1st fraction.	2nd fraction.	3rd fraction.	4th fraction.	5th fraction.		
$MgNH_4PO_4.6H_2O$ obtained per gm. of nucleic acid.	0.625	0.623	0.617	0.625	0.652	0.617		
Calculated half.	0.313	0.312	0.309	0.313	0.326	0.309		
Magnesium correction.	0.018	0.017	0.017	0.015	0.010	0.003		
Values found in preceding tables.	0.295	0.295	(0.292)	(0.298)	0.316	0.306		
Maximum moisture.	8.5	6.3	7.6	6.0	3.9	8.3		
Phosphorus percentage calculated for the dry substance.	8.67	8.45	8.48	8.44	8.61	8.55	8.60	9.52

reasonably be accepted, the preparations of nucleic acid were heated at 105° until the appearance of a pale yellow color showed that decomposition had begun. The total loss in weight was assumed to be moisture and was used as a basis for calculating the phosphorus of the dry preparations. High results were also secured by neglecting the magnesium correction in calculating the total phosphorus, although the magnesium impurity may well be magnesium phosphate. The phosphorus percentages agree closely with one another (8.67, 8.45, 8.48, 8.61, 8.55) and with the one reported by Levene[2] (8.60), but differ from that required by the formula for yeast nucleic acid (9.52).

ABSTRACTS OF SCIENTIFIC PROCEEDINGS.

ON FAT ASSIMILATION.

By W. R. BLOOR.

(*From the Laboratory of Biochemistry, Harvard Medical School, Boston.*)

In a previous communication[4] evidence has been presented to show that the lecithin of the whole blood increases during fat absorption. The present paper is a report of further work along the same line in an attempt to locate the point of lecithin production. For this purpose dogs were given a feeding of olive oil and then blood samples were taken at once and every 2 hours after for 8 hours. Analyses were made for total fatty acids, cholesterol, and lecithin in both whole blood and plasma, from which, knowing the percentage of corpuscles in the blood, the content of the corpuscles in each of the above constituents was calculated. Eight experiments on two dogs were carried out. The results showed: (1) as regards total fatty acids, increases in both plasma and corpuscles, being greater in the latter and indicating that the corpuscles take up the absorbed fat from the plasma; (2) as regards cholesterol, no notable changes; (3) as regards lecithin, a great increase in the corpuscles with a slight increase in the plasma. The results are believed to show (1) that lecithin is the first step in the transformations of the fats in metabolism and (2) that the lecithin formation takes place in the corpuscles.

FAT INFILTRATION OF THE CAT'S KIDNEY.

By V. H. MOTTRAM.

The fatty acids of organs, such as the liver, heart, and kidney, are well known to be more unsaturated than those found in the fat in other parts of the body. And in cases investigated up to the present it is found that the iodine value of the fatty acids of the liver is almost invariably between that of the fatty acids of lipoid tissue and that of heart and kidney fatty acids. The only exception is that of the fatty acids of the cat's

[4] Bloor, W. R., *J. Biol. Chem.*, 1915, xxiii, 317.

kidney.[5] The subject has been reinvestigated as follows. Cats were kept in quiet surroundings on a constant liberal diet of oatmeal, milk, and meat for at least a month. They were then killed by bleeding under narcosis and estimations were made of percentage and iodine values of the fatty acids of the liver and right and left kidneys, and of the iodine value of the dépôt fatty acids. Histological preparations of the kidneys were also made.

The results of the experiments were (1) that the iodine values of the fatty acids of the kidneys never reached the level of the liver fatty acids; (2) that in some cases they were slightly above those of the fatty acids of lipoid tissue; (3) that in others they were strikingly below the latter, in which case the fatty acids were fluid at room temperature; (4) that the iodine values of the fatty acids were inversely proportional to the percentage present, suggesting an infiltration with milk fatty acids (iodine value 20 to 30); and (5) that this interpretation was borne out by histological preparations. In the latter, where there was much fat present, it was invariably in the convoluted tubules and rarely in the straight tubules; in some cases fat could be made out in the blood vessels (lipemia) and in others in the lumina of the convoluted tubules (lipuria?). Nuclear chromatin was clear and stained sharply.

Conclusions to be drawn from this research are that (1) desaturation of cat's kidney fatty acids much beyond that of the fatty acids of lipoid tissue is not normal (contrast with man, dog, goat, pig, rabbit); *i.e.*, if cat's kidneys metabolize fatty acid as a source of chemical energy they can utilize normal fatty acids; and (2) in the presence of fat in the food and blood stream, cat's kidneys show a marked infiltration with fat.

THE COMPOSITION OF ADIPOCERE.

By R. F. RUTTAN and M. J. MARSHALL.

The tabulated results of the quantitative analysis are as follows:

	per cent
Palmitic acid	67.52
Stearic acid	3.3

[5] Leathes, J. B., *Lancet*, 1909, i, 95.

	per cent
Oleic acid	5.24
ι-Hydroxy stearic acid	9.48
κ-Hydroxy stearic acid	6.32
Stearin and palmitin	1.21
Olein	0.16
Unsaponified matter	0.87
Calcium soaps	4.41
Protein	0.665
Ash	0.578
Humus and undetermined	0.247
	100.000

The fatty acids and traces of fats, etc., soluble in ether constituted 94.1 per cent of the adipocere and gave the following physical and chemical constants:

Specific gravity at 100°C	0.8436
Refractive index at 60°	1.436
Melting point	60–63°C.
Acid value	201.7
Saponification value	207.0
Iodine value	6.04
Acetyl value	34.75

The total nitrogen present was only 0.1778 per cent, of which only 0.0289 per cent was ammonia nitrogen.

As a result of the studies recorded in this paper we are justified in concluding that adipocere is the residue of the preexisting fats of animals. It is composed almost entirely of the insoluble fatty acids left after the slow hydrolysis of the fats in wet ground. The protein matter has entirely disappeared and the glycerol, soaps, etc., resulting from the hydrolysis, are carried away in aqueous solution.

The insoluble hydroxy stearic acids which are so characteristic of adipocere are derived from a portion of the oleic acid in the original fat by hydration.

No margaric acid or hydroxy margaric acid is present. Ammonium and other soluble soaps are absent and only the calcium salts are present in small quantity in a matured specimen of adipocere such as that reported upon.

ON THE RÔLE PLAYED BY ELECTROLYTES IN DETERMINING THE PERMEABILITY OF PROTOPLASM.

By G. H. A. CLOWES.

(*From the Biological-Chemical Laboratory of the State Institute for the Study of Malignant Disease, Buffalo.*)

If aqueous solutions of soap or caustic soda are prepared sufficiently concentrated to give a system of fifty drops when passed through olive oil from a Traube stalagmometer, any increase in the amount of NaOH or soap causes a considerable increase in the number of drops, and any decrease causes a reduction. Similarly salts of monovalent cations, Li, Na, K, etc., cause an increase in the number of drops, while salts of divalent and trivalent cations, Ca, Sr, Ba, Fe, Al, etc., cause a reduction. Furthermore, when salts of monovalent cations are employed in conjunction with salts of divalent cations, they are found to balance one another or exert a compensatory effect upon one another in certain ratios, which are those most commonly observed in studying the antagonistic effects of various salts upon one another in biological systems. NaCl, for example, is balanced by means of $CaCl_2$ in molecular ratios varying from one to two of the latter to 100 of the former, according to concentration. While alkalies, and tri- and divalent anions cause an increase in the number of drops, acids, up to a certain concentration, and tri- and divalent cations cause a reduction in the number of drops. Since a soap film is formed at points of contact between oil and water, the number of drops formed is an index of the resistance of the film in question, or the readiness with which it is dispersed or peptized by the constituents of the aqueous phase.

It appears, therefore, that electrolytes may be divided readily into two classes; those which promote dispersion of the soap film and consequently increase its permeability, and those which promote aggregation of the soap film and consequently diminish its permeability. It is interesting to note that these substances exert effects both qualitatively and quantitatively on soap films which correspond with those observed by Loeb, Lillie, Osterhout, and others, while studying the antagonistic effects exerted by electrolytes on living cells. Salts of Mg and anesthetics, when employed in suitably constituted systems, exert at certain opti-

mum points a maximum protective effect upon the film, but when employed at higher concentrations exert a destructive effect upon the film, and the concentrations at which these effects are produced correspond very closely with those recorded by Lillie and others in studying the protective effect exerted by anesthetics on marine organisms.

In upwards of 100 experiments parallel results have been obtained in physical and biological systems, even to the extent of observing substances like Mg functioning in one case as an antagonist to calcium in ratios of 2 : 1, and in another case exerting the same effect as calcium when functioning as an antagonist to NaCl in ratios of one or two to 100. On the basis of these experiments a theory has been developed regarding the equilibrium of protoplasmic systems, further details regarding which will be published shortly, based on the assumption that adsorbed ions, by influencing the surface tension of concentration films promote or retard the dispersion of these films in the external aqueous medium surrounding protoplasm, and thus promote or retard the intermittent intercommunication between the interior aqueous phase of protoplasm and the exterior aqueous medium, which may be assumed to take place normally under the influence of varying concentrations of OH resulting from varying concentrations of CO_2 and other substances produced in the course of normal metabolism.

A COMPARISON OF THE SÖRENSEN, VAN SLYKE, AND COLORIMETRIC METHODS FOR THE ESTIMATION OF PROTEIN HYDROLYSIS.

By VICTOR JOHN HARDING and REGINALD M. MacLEAN.

A study was made of the hydrolysis of various proteins by pancreatic extracts, and the proteoclasis was followed by the Sörensen formaldehyde titration method, the Van Slyke gasometric method, and the authors' new colorimetric method. The last depends upon the quantitative application of the ninhydrin reaction. The representative proteins used were: serum albumin and globulin, gluten, fibrin, gelatin, casein, nucleoprotein, and two samples of peptone.

The results uniformly showed that the Van Slyke and the authors' methods gave identical results which were higher than those given by the Sörensen method. A discrepancy between the results of the colorimetric and gasometric methods was sometimes observed, however, when applied to native proteins, the colorimetric method giving lower figures. This was believed to be due to the reactivity of the ninhydrin reaction being confined chiefly to α-amino groups.

THE DISTRIBUTION IN FOODS OF THE SO CALLED VITAMINES AND THEIR ISOLATION.

By M. X. SULLIVAN and CARL VOEGTLIN.

(From the Pellagra Hospital, United States Public Health Service, Spartanburg, S. C.)

Antineuritic substance or substances were found in wheat bran, in the common black-eyed cow-pea of the Southern States, and in ox liver, but not in pork fat. In isolating the vitamine fraction, various modifications of Funk's methods were tried. The most satisfactory method is as follows: The hot alcohol-soluble residue was hydrolyzed 5 hours at 90°C. with 5 or 10 per cent H_2SO_4 in a current of CO_2. The cold filtrate was precipitated by phosphotungstic acid. The resulting precipitate was decomposed either by rubbing with neutral lead acetate, or by dissolving the precipitate in 50 per cent alcohol and treating the solution with excess of lead acetate. The filtrate from the lead precipitate was freed from Pb by H_2S and concentrated *in vacuo* at 50°C. The solution filtered was precipitated with considerable excess of silver acetate. The filtrate was made slightly alkaline with $Ba(OH)_2$. The resulting precipitate, suspended in water and made slightly acid with H_2SO_4, was decomposed by H_2S. The AgS filtrate was concentrated *in vacuo* to a small volume and brought to a slight acidity by means of dilute Na_2CO_3. The method here given avoids the possible destructive action of $Ba(OH)_2$ in the decomposition of the phosphotungstic precipitate and the oxidizing action of silver nitrate in the precipitation of the vitamine fraction.

By treating the alcohol residue of liver and peas with water and extracting with ether an aqueous solution can be obtained

free from lecithin. This aqueous solution on hydrolysis yielded a crude antineuritic preparation which was found non-toxic in moderate doses.

Chickens fed on corn-bread made from corn-meal, milk, and baking soda (sodium bicarbonate) rapidly developed polyneuritis, while those fed on corn-bread made from corn-meal, milk, and sodium chloride remained in good condition.

THE RELATION OF LIPOIDS TO VITAMINES.

By M. X. SULLIVAN and CARL VOEGTLIN.

(From the Pellagra Hospital, United States Public Health Service, Spartanburg, S. C.)

On extracting wheat bran with 90 to 95 per cent alcohol and removing the alcohol *in vacuo* at 50°C. a brownish yellow lipoid mass is obtained. This lipoid mass is soluble in chloroform and ether but has little solubility in cold water. The crude material possesses little, if any, curative power when given to fowl with polyneuritis, but after hydrolysis with acid and proper fractionation a vitamine fraction possessing strong antineuritic properties is obtained.

In the presence of considerable water, however, ether extraction of the lipoid mass yielded three layers, an ethereal layer, a middle semisolid layer, and an aqueous layer.

When these layers were separately hydrolyzed and fractionated to the vitamine fraction only the aqueous layer yielded a fraction possessing antineuritic properties.

It is obvious that if vitamine-containing foodstuffs rich in fat and lipoids and practically free from water be extracted with lipoid solvents, such as chloroform or ether, the erroneous conclusion might be drawn that the antineuritic substance or substances are constituents of lipoids *per se*.

In reality the antineuritic material is either dissolved in, or mixed in the lipoid or is in easily separable combination with it.

A SIMPLIFIED PROCEDURE FOR THE DETERMINATION OF THE CARBON DIOXIDE TENSION IN THE ALVEOLAR AIR.

By W. McK. MARRIOTT.

(From the Department of Pediatrics, Johns Hopkins University.)

A modification of the Plesch method for the collection of air samples is described. A simply constructed mask renders the collection possible with infants and with laboratory animals.

The carbon dioxide tension in the sample is determined by bubbling the air through a 0.01 N solution of sodium bicarbonate containing phenolsulphonephthalein as an indicator. The reaction of the solution and the corresponding color obtained are functions of the carbon dioxide tension. The color is compared with that of standard solutions of mixed phosphates containing the indicator, made up in such a way that the tension of carbon dioxide in mm. may be directly read off. These standard solutions are practically permanent. Slight changes in temperature are without effect on the accuracy of the method. The temperature, however, may be easily controlled by immersing the tubes in a beaker of water at the temperature selected as a normal (20°C.) Changes in the barometric pressure are, from the nature of the method, entirely without influence.

Results invariably agree closely with those obtained by the analysis of the air sample in the usual Haldane gas analysis apparatus.

THE USE OF A NEW REAGENT FOR MICROCOLORIMETRIC ANALYSIS AS APPLIED TO THE DETERMINATION OF CALCIUM AND OF INORGANIC PHOSPHATES IN THE BLOOD SERUM.

By JOHN HOWLAND, F. H. HAESSLER, and W. McK. MARRIOTT.

(From the Department of Pediatrics, Johns Hopkins University.)

The methods described are based on the fact that the red color of a solution of ferric thiocyanate is discharged by certain substances, among which are oxalates and phosphates.

Calcium is precipitated as the oxalate, dissolved in acid, added to a standard solution of ferric thiocyanate, and made up to a definite volume. The color of the resulting solution is compared

with that of a solution containing known amounts of calcium oxalate and ferric thiocyanate. The phosphates are precipitated as a magnesium and ammonium phosphate. The precipitate is dissolved and color comparisons are made as above.

The calcium content of the serum in children was found to be normal or but slightly diminished in most cases of rickets studied. When the rickets was accompanied by evidences of tetany the calcium content of the serum was very low. In dogs there was found a progressive diminution in the calcium of the serum, following parathyroidectomy.

Normal human serum contains very small amounts of inorganic phosphates. The amount, however, has been found to be markedly increased in certain conditions in infants accompanied by acidosis and characterized by diminished renal function. In these cases acid phosphate is apparently retained through failure of the excretory activity of the kidney.

The principle utilized in the methods described is applicable to the determination of other substances, especially lactic acid, and is at present being used for that purpose.

CONTINUOUS INTRAVENOUS INJECTIONS AT UNIFORM RATES.

By W. D. SANSUM, RUSSEL M. WILDER, and R. T. WOODYATT.

(*From the Otho S. A. Sprague Memorial Institute Laboratory for Clinical Research, Rush Medical College, Chicago.*)

A demonstration is given of a motor-driven quantitative pump for continuous intravascular administrations, perfusions, etc., at controllable rates, with a discussion of its application in the study of quantitative aspects of absorption, metabolism, and secretion, and details of experiments in which the method was applied in the study of glucose, galactose, levulose, lactose, and glyceric aldehyde.

The intravenous tolerance limit expressed as a velocity has been found for glucose at close to 0.85 gm. per kg. of body weight hourly, for levulose close to 0.15 gm., for galactose and glyceric aldehyde close to 0.1 gm., for lactose close to 0.0 gm. per kg. per hour. Glucose may be given by vein to normal resting rabbits, dogs, or men at rates below 0.85 gm. per kg. per hour for 2 to 12 hours, with no glycosuria and no acceleration of diuresis.

At rates of injection above this limit, glycosuria and diuresis appear. The rate of glycosuria becomes constant at a level characteristic for each uniform injection rate, provided all experimental conditions are appropriate. It may require 4 to 5 hours for the excretions to become constant with higher injection rates. * Observations were presented on sugar-, salt-, and fever-diuresis and water balance.

THE PRODUCTION OF HYPERGLYCEMIA AND GLYCOSURIA BY MAGNESIUM SALTS.

By I. S. KLEINER and S. J. MELTZER.

(*From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.*)

In their experiments on the action of magnesium salts, Meltzer and Auer[6] observed that after subcutaneous injections of magnesium sulfate the urine of rabbits contains a reducing substance. Underhill and Closson,[7] who later noticed the presence of hyperglycemia after an intravenous injection of magnesium sulfate, ascribed the hyperglycemia to the asphyxia which the magnesium salts produced in their experiment.

In a series of experiments which we have recently carried out on dogs, all the animals had from the beginning to the end of the experiment either intratracheal insufflation or the usual artificial respiration. The occurrence of asphyxia was thus excluded. The operative part was done under local anesthesia. In most of the experiments a $\frac{M}{4}$ solution of magnesium sulfate was injected intravenously. There was a considerable increase of the sugar content of the blood after infusion in practically all experiments. In most cases the original glycemia did not exceed 0.13 per cent, while at the end of the injection or some time later, the sugar content of the blood was often as high as 0.4 per cent and even higher. In blood taken about $1\frac{1}{2}$ hours after the end of an injection the glycemia was often found to have dropped to the original content.

There can be no doubt that magnesium sulfate produces considerable hyperglycemia which is not due to asphyxia; it is

[6] Meltzer, S. J., and Auer, J., *Am. J. Physiol.*, 1905, xiv, 371.

[7] Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1906, xv, 321.

CORRECTION.

On page xx, Vol. XXIV, No. 3, lines 6 and 7 should read *Observations were presented on sugar and salt fevers, diuresis, and water balance.*

produced in some way specifically by the magnesium salt. (In an experiment with sodium sulfate no hyperglycemia was produced.)

It is a noteworthy fact that the glycosuria was very little marked and not in proportion to the hyperglycemia. Glycosuria was often entirely absent and when present it never reached even 0.5 per cent. The intravenous injection of magnesium sulfate also produced very little diuresis.

EXPERIMENTAL AND CLINICAL STUDIES ON MENTAL DEFECTIVES.

II. THE GLYCOSURIC REACTION OF INSTITUTION INMATES IN RELATION TO NUTRITIONAL AND PATHOLOGICAL CONDITIONS.

By AMOS W. PETERS.

With the Assistance of Mary E. Turnbull and Caroline D. Blackburn.

(*From the Biochemical Laboratory, Training School, Vineland, N. J.*)

Feeble-minded inmates of an institution were tested for dextrose tolerance with the result that the nutritional factor had a more general significance than the pathological. The method permitted the estimation of 0.01 per cent of sugar in clarified urine. The degree of tolerance was expressed as:

$$\text{Saturation capacity} = \frac{\text{Sugar ingested, gm.}}{\text{Body weight, kg.} \times \text{sugar excreted, gm.}}$$

The number of units obtained shows the capacity of the organism to absorb sugar before the renal overflow occurs.

The age group of 5 to 12 years, of both sexes, showed a high saturation capacity of 269 units compared to 96 units for the age group 13 to 37 years, with low tolerance of 61 units for females in the latter group. Repetitions showed a high degree of constancy. There was a low tolerance group of 49 units, mostly of "disturbed" females, and a Mongolian group showing high tolerance of 205 units. The only pathological groups found were the low tolerances and the Mongolians, excessively high toler-

ance being the rule with 72 per cent of the cases. The notably high dosages required for the cases as a whole were due to chronic undernutrition and defective institutional diet.

THE DETERMINATION OF CALCIUM IN BLOOD.

By JOHN O. HALVERSON and OLAF BERGEIM.

(*From the Department of Physiological Chemistry of Jefferson Medical College, Philadelphia.*)

A method was suggested for the determination of calcium in whole blood, plasma, or serum. Ordinarily about 10 cc. of whole blood were used. It was deproteinized by means of picric acid in strongly acid solution and the calcium precipitated as oxalate directly in the filtrate according to McCrudden's procedure. The precipitate was washed by centrifugation and the oxalate titrated with 0.01 N permanganate solution under standardized conditions.

Magnesium was determined in the filtrate after destruction of ammonium salts. The precipitation as magnesium ammonium phosphate and the washing of this precipitate were carried out in centrifuge tubes. The ammonia content of the precipitate was determined colorimetrically after distillation or by titration using methyl red as an indicator. This was found preferable to the nephelometric determination of the phosphorus content except where less than 2 cc. of blood were available.

A COMPLETE METABOLISM STUDY OF GOITER WITH THE EFFECT OF THYROID AND THYMUS TREATMENT.

By JOHN O. HALVERSON, OLAF BERGEIM, and PHILIP B. HAWK.

(*From the Department of Physiological Chemistry of Jefferson Medical College, Philadelphia.*)

The metabolism of nitrogen, phosphorus, sulfur, calcium, and magnesium was studied in a case of exophthalmic goiter which appeared to respond readily to thyroid and thymus treatment.

The study was made in five periods of 5 days each: (1) a control period; (2) a period with thyroid treatment; (3) a control period; (4) a period with thymus treatment; (5) a control period. The patient was on a constant food and water intake throughout.

In Period 1 there was a marked loss of nitrogen, phosphorus, and magnesium, while the sulfur and calcium were practically balanced. On thyroid treatment marked retentions were observed for all the elements, the retention reaching a maximum for nitrogen, sulfur, and phosphorus in the final period and for calcium and magnesium in the third period. These retentions continued throughout the experiment and indicate a stimulation of metabolism by thyroid. Thymus treatment, however, appeared to depress this stimulating effect as higher retentions were observed in the post-thymus than in the thymus period. Thyroid treatment led to diuresis while on thymus treatment the urine volumes decreased greatly. Likewise the body weight decreased considerably on thyroid treatment and rose again on thymus administration. The fluctuations in weight could be accounted for on the basis of water loss and nitrogen elimination.

ORIGIN AND DETERMINATION OF CREATINE IN MUSCLE.

By L. BAUMANN, H. HINES, and J. MARKER.

(*From the Laboratories of Internal Medicine and Physiology, Iowa State University, Iowa City.*)

The figures obtained for creatine in muscle by the Janney and Blatherwick extraction method agree closely with those obtained by the acid hydrolysis method reported by Baumann two years ago. The older method has been simplified so that a complete determination may be carried out in less than half an hour after the extract is obtained. It may also be used to advantage in the determination of creatine in organs. All the substance in the hydrolyzed muscle extract responsible for the Jaffé reaction may be precipitated by picric acid and potassium.

Arginine, betaine, methylureidoacetic acid, sarcosine plus urea, and choline plus urea have been perfused through muscle tissue. Only choline plus urea consistently lead to an increase in muscle creatine.

IS THE GLUCOSE RETAINED, WHEN SODIUM CARBONATE IS ADMINISTERED TO DEPANCREATIZED DOGS, STORED AS GLYCOGEN?

By B. KRAMER and J. MARKER.

(*From the Physiological Laboratory of the State University of Iowa, Iowa City.*)

The experiments here reported are a few of a series covering a period of several years in which the object was to determine what happens to the glucose retained when sodium carbonate is administered to depancreatized dogs. The well known fact that in normal animals, both cold- and warm-blooded, sodium carbonate inhibits glycogenolysis, made the assumption plausible that the ability of sodium carbonate to lower the glycosuria and the D: N ratio in diabetic dogs might be attributed to some effect upon the glycogenetic or glycogenolytic mechanism.

Two distinct methods of procedure were adopted. First, a totally depancreatized dog was fed on meat and sodium carbonate until the urinary analysis showed that a considerable quantity of glucose had been retained. The animal was then quickly anesthetized, specimens of liver and muscle were removed, and the glycogen content was determined, and only traces were found. To corroborate this result, a similar diabetic animal was rendered glycogen-free by repeated injections of adrenalin. Then meat and carbonate were fed until considerable glucose, which must have come from protein, had been retained. The adrenalin administration was then resumed, and the failure to demonstrate extra sugar in the urine was interpreted as indicating that none of the glucose retained had been stored as glycogen. The saliva was frequently tested for glucose, and only minute traces were found. Gastric and intestinal washings failed to show a sufficient amount of reducing substances to account for more than a very small fraction of the glucose retained even when the sugar content of the blood must have been very high, as a result of combined effects of ether anesthesia, the operation, and the intravenous injection of a solution of glucose.

Conclusions.—Sugar retained when sodium carbonate is administered to depancreatized dogs is not stored as glycogen nor is it eliminated by way of the saliva or the gastro-intestinal tract.

THE INFLUENCE OF ALKALI ON THE DIABETES OF PARTIALLY AND TOTALLY DEPANCREATIZED DOGS.

By J. R. MURLIN and B. KRAMER.

(From the Physiological Laboratory of the Cornell University Medical College, New York City.)

Continuing the observations, reported to this society one year ago, on the influence of sodium carbonate on the glycosuria, the hyperglycemia, and the respiratory metabolism of depancreatized dogs, we have to report today confirmation of the statements then made in the following particulars: (1) disappearance of sugar from the urine of a totally depancreatized animal within 4 hours following the infusion of 300 cc. of Ringer's solution containing 1 per cent Na_2CO_3; (2) no increase in the blood sugar accompanying this; (3) a rise of the respiratory quotient to 0.90, 0.85, and 0.85 in successive hours following the administration of Na_2CO_3 with glucose intravenously to a dog whose urine had been rendered sugar-free even while on full diet by the administration of sodium carbonate.

Of new observations we have the following to report: (1) no rise in R. Q. after the administration of Na_2CO_3 to normal dogs; (2) a limited capacity to oxidize glucose on the part of a dog in which a sufficient amount of pancreas had been left (at the main duct) to prevent glycosuria on a meat diet; (3) an increase in this capacity, as judged by the rise in R. Q. and an increase in heat production, when Na_2CO_3 was given with glucose, but not when Na_2CO_3 was given alone; (4) a marked rise in R. Q. when NaOH, in amount to make 0.1 N, added to Ringer's solution was administered intravenously to a totally diabetic dog on the 5th day following pancreatectomy.

The complete reports will appear in an early number of this *Journal.*

SOME TESTS OF THE DIGESTIBILITY OF KAFIR-CORN AND INDIAN CORN-MEAL PREPARED FOR THE TABLE IN THE USUAL WAY.

By C. F. LANGWORTHY and A. D. HOLMES.

(From the Office of Home Economics, States Relations Service, United States Department of Agriculture.)

In certain areas of the United States, too dry for the most successful cultivation of the better known food grains, the grain sorghums will flourish. The result is that much study has been made of the cultivation and possible uses of Kafir-corn, feterita, milo-maize, and other more or less similar crops. They are now well recognized foodstuffs in some other countries and have at least a limited use in the United States.

From Kafir-corn a meal is made which has many uses. To some palates, at least, it resembles buckwheat in flavor. Like corn-meal, it is not suitable alone for yeast-raised bread, owing to a lack of gluten, but can be used for making many other types of bread and similar foods familiar to every housewife who uses corn-meal. In view of these facts, it was of interest to the Department of Agriculture to include the grain sorghums in its studies of the food value and possible uses of agricultural products.

As the result of seven experiments with healthy young men as subjects, it appeared that Kafir-corn bread, when eaten as a part of a simple mixed diet, had, on an average, a digestibility of 46 per cent for protein and 97 per cent for carbohydrates. In other tests, under somewhat different conditions, the values for the digestibility of protein were a little lower.

For comparison, tests were made, under like experimental conditions, with corn-meal ground to the same degree of fineness as the Kafir-corn. The coefficients of digestibility of corn bread as shown by the average of three tests, were: protein, 55 per cent; carbohydrates, 97 per cent.

The composition and thoroughness of digestion, as well as the economic considerations involved, indicate that such grains as Kafir-corn are worthy of serious consideration as foodstuffs.

The experiments briefly cited form a small part of an extended study of grain sorghums and their uses, the results of which await publication.

THE DETERMINATION OF β-HYDROXYBUTYRIC ACID.

By P. A. SHAFFER and R. S. HUBBARD.

(From the Laboratory of Biological Chemistry, Washington University, St. Louis.)

A further revision of the conditions underlying the oxidation of β-hydroxybutyric acid by potassium bichromate and sulfuric acid has shown that the oxidation proceeds faster with increasing concentration of sulfuric acid, and at a concentration of about 10 N the oxidation may be completed in about 20 minutes. If the bichromate is added in dilute solution only as fast as it is reduced the yield of acetone is about 90 per cent of the amount theoretically formed from the hydroxybutyric acid present. Crotonic acid is not formed until the concentration of sulfuric acid reaches about 12.5 N. Based on these facts the procedure for the determination of hydroxybutyric acid may be greatly shortened in time without sacrifice in accuracy. Further modifications were given for the determination of acetone bodies in urine, blood, and tissues.

The authors were unable to confirm the claim of Folin and Denis that the oxidation of hydroxybutyric acid by the modified procedure described by them yields theoretical amounts of acetone; about 85 to 90 per cent is the maximum amount obtained under their conditions.

HYDROGEN ION CONCENTRATION IN AUTOLYSIS.[8]

By MAX MORSE.

Thymus was used for the experiments. Digests *in vitro* were conducted and the concentration of hydrogen ions was estimated at regular intervals for a period of 10 days by means of the Sörensen method for colorimetrically determining P_H. The initial determination gave $P_H = 6.4$. At the time when the curve of rate of autolysis as measured by the non-precipitable nitrogen reached its approximate maximum, the curve of P_H had done likewise ($P_H = 4.2$). Throughout, the two curves ran similarly, so that the conclusion seems justified that we are deal-

[8] Read by title.

ing with an autocatalytic phenomenon, wherein the acidity, due to lactic acid, phosphate, etc., acts as a true co-enzyme or catalyzer to accelerate the process, or there may be a change of physical state, such as Jobling and others have postulated for the enzyme actions studied by them. There is no evidence that there is any modification of substrate, on the part of the acid, rendering certain protein components digestible for the tissue enzyme.

CREATINE IN ATROPHY.[8]

By MAX MORSE.

Creatine estimations have been made on a number of children with various diseases involving atrophy, such as poliomyelitis, marasmus, recurrent vomiting, nephritis, lobar pneumonia, etc. Retention of creatinine has been observed in all nephritic cases and also in those of recurrent vomiting, wherein virtually a starvation condition results. One case of marasmus was followed through its entire range and it was clear that one could follow the patient's condition by the curve of creatinine-creatine ratio. In one case, turpentine nephritis, no creatinine could be determined in the urine for several days by any method used. In all cases without exception, creatine ran high, especially in the instances of anterior poliomyelitis, where a noticeable amount was excreted daily. Practically no work has been done upon the problem of correlating creatinine and creatine changes with pathologic states, and the value of such studies, theoretically and clinically, has been demonstrated by these studies.

The studies have been conducted in common with Dean Cutter of the University of Nebraska College of Medicine and the Child Saving Institute, Omaha.

GROWTH ON STRICTLY VEGETABLE DIETS.[8]

By E. B. HART and E. V. McCOLLUM.

We have published data from this laboratory showing that omnivora will not grow normally on certain grains or mixtures of grains unless there are added distinct supplements such as salts, proteins, accessories, or combinations of these. A number of

investigations by other workers have also been reported in the literature, supporting the view that strictly vegetable diets will not be sufficient for the growth and normal physiological condition of the organism. In fact the view that a strictly vegetable diet is insufficient has been emphasized from time to time by students of human nutrition. In contrast to this view are the obvious results of the complete development of herbivora on strictly vegetable diets. Guinea pigs, sheep, cattle, etc., often make 80 per cent of their growth from such sources.

It appeared probable, therefore, that the roughage or that part of the plant other than the seed was the material capable of supporting growth when fed alone or acting as a supplement to grains and grain mixtures. Failure of early experiments to maintain growth with grains and small daily allowances of green vegetable tissue is to be explained on the basis of an insufficiency in quantity of the stem or leafy part of the plant in the diet. The herbivora consume daily large masses of these materials.

In our own experiments, omnivora (swine and rats) maintained sustained growth and apparent physiological vigor when the grains were intimately mixed with a roughage so as to insure a liberal consumption of the latter. With 15 or 25 per cent of alfalfa meal in grain rations we have succeeded in maintaining growth and well-being in swine, whereas without this roughage complete failure results. The probable factors contributed by the roughage will be discussed in a future paper presenting these data.

SOME DIASTASE ACCELERATORS.

A PRELIMINARY REPORT.

By E. W. ROCKWOOD.

(*From the Chemical Laboratory of the University of Iowa, Iowa City.*)

The action of amino compounds was tested on the salivary and pancreatic amylases. There was a marked increase in the formation of reducing sugars in the presence of glycine, hippuric acid, and acetamide. Anthranilic acid and urea had no effect. The effect of the amino compounds is most evident when very small quantities of the ferments are used. The work is being continued.

UREASE CONTENT OF CERTAIN BEANS, WITH SPECIAL REFERENCE TO THE JACK BEAN.

By J. G. MATEER and E. K. MARSHALL, Jr.

(From the Laboratory of Pharmacology, Johns Hopkins University, Baltimore.)

A number of beans have been examined for urease. *Phaseolus calcaratus, Phaseolus angularis, Phaseolus aconitifolius, Phaseolus Mungo,* early Florida velvet bean, Chinese velvet bean, *Vigna sinensis, Dolichos Lablab, Stizolabium deeringianum,* do not contain the enzyme, whereas in *Phaseolus aureus, Lupinus albus, Dolichos biflorus,* sword bean, and jack bean it is present.

There exists great variability in the urease content of beans. The jack bean contains about fifteen times, and the sword bean about five times as much urease as the soy bean, while *Dolichos biflorus* contains about $\frac{1}{5}$, the white lupine about $\frac{1}{50}$, and *Phaseolus aureus* about $\frac{1}{350}$ as much as the soy bean. The aqueous extract of the jack bean does not decompose ereptones, casein, hippuric acid, leucyl-glycine, uric acid, creatinine, creatine, adenine. The jack bean aqueous extract contains about 25 mg. of solid material, while the aqueous extract of the soy bean contains about 40 mg. per 1 cc.

CONCERNING PROTEIN SYNTHESIS AND METABOLIC DISEASES

By N. W. JANNEY.

(From the Chemical Laboratory of the Montefiore Home and Hospital for Chronic Invalids, New York.)

There exists considerable indirect evidence that carbohydrates contribute material which enters into the synthesis of protein. The sparing action of glucose on protein, which cannot be adequately accounted for on dynamic grounds, may be due to protein repair and synthesis from intermediary products of glucose metabolism.

The glands of internal secretion promote growth, *i.e.*, the formation of new protoplasm, and are indispensable for the maintenance of its nutritional processes. These organs also regulate the distribution of carbohydrates in the organism. In view of the possible synthetic function of glucose, the ductless glands may

therefore function to regulate the supply of material for the synthetic repair, regeneration, and formation of living protein. Recent experiments by the writer can be adduced to support this hypothesis. It was shown that the sparing action of glucose on protein was increased by the additional feeding of minute doses of thyroid tissue.

If the synthesis of protein from carbohydrates should prove to take place extensively a change of view-point in regard to various metabolic problems might be necessary. The abnormalities of protein metabolism in thyroid disease accompanied by disturbances of carbohydrate metabolism, could be reasonably explained. The same holds true for many phenomena of diabetes. Aside from the glycosuria and accompanying symptoms, this disease is characterized by loss of protein to the organism, degenerative lesions and trophic symptoms indicating a disturbance of protoplasmic nutrition. These anomalies in diabetic metabolism may be due to an inadequate supply of synthetic intermediate substances normally produced from carbohydrates.

ENZYMES OF A CELLULOSE-DESTROYING FUNGUS FROM THE SOIL, PENICILLIUM PINOPHILUM.[8]

By E. D. CLARK and F. M. SCALES.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

The bacterial and fungus flora of soils is active in causing decomposition in both plant and animal matter. Scales[9] has shown that the soil fungus *Aspergillus terricola* contains enzymes acting upon many substances of biological origin. *Penicillium pinophilum* is one of the most active destroyers of cellulose yet isolated from soils, while *Aspergillus terricola* contains little if any cytase.

In general, the methods of culture and of preparing the enzyme powder used by Scales were applied also in our study of this *Penicillium*. The fungus mats on the surface of the nutrient solutions were prepared for the tests by the *Acetonedauer* treatment. A modified neutral Raulin's solution was used, ammonium sul-

[9] Scales, F. M., *J. Biol. Chem.*, 1914, xix, 459.

fate or peptone being employed as a source of nitrogen. Pure cellulose (acid-washed filter paper) was supplied as a source of carbon. The fungus often produced a dense mat on the surface of the liquid, the under side of this mat showing bright crimson spots. The cellulose cultures were incubated at about 28°C. for 2 to 3 weeks. During this time the cellulose had been changed so that it appeared soft and pulpy, the individual fibers being frayed out, separated, and transparent.

Some of the enzyme preparations made by the acetone treatment were from cultures containing purely inorganic nutrients, some from media containing peptone, while one was allowed to autolyze for 5 days with toluene as an antiseptic. Our preparations were gray or brown in color and the most active one contained 4.6 per cent of nitrogen. Definite amounts of the enzyme powders were weighed, mixed with distilled water, and allowed to stand for a time before being tested for their activity. Boiled enzyme solutions were used in every case as controls. The following enzymes were found to be especially active: sucrase, emulsin, and amidase; while diastase, catalase, and lipase were not as active. Tests for peptic, tryptic, ereptic enzymes, and also peroxidase, gave negative results. However, we should not conclude from this that proteolytic enzymes are absent in the growing fungus, because it is not likely that an active organism of this sort would be unable to attack protein. The activity of sucrase, diastase, lipase, and amidase was determined by quantitative methods upon various preparations of the enzyme powder. Space does not permit the inclusion of the results of our quantitative determinations in this study.

The most active preparation was made from the growth for 3 weeks on the inorganic nutrient solution containing ammonium sulfate and cellulose as the source of nitrogen and carbohydrate, respectively. Furthermore, this culture was just at the stage of active spore formation. In many cases the culture fluid from the older cultures showed a weak enzyme activity, indicating that some of the enzymes had diffused out of the cells. A study of the cytase of this organism was impossible at the time owing to the lack of methods for determining small quantities of cellulose with accuracy.

THE PROTEIN OF THE JACK BEAN CANAVALIA ENSIFORMIS.[8]

By CARL O. JOHNS and D. BREESE JONES.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

The globulin was obtained by extracting with distilled water or dilute sodium chloride solutions and dialyzing the filtered solution in running water. The average figures for five different preparations are, carbon 52.36, hydrogen 6.90, nitrogen 16.29, sulfur 0.40, oxygen 24.05 per cent. When an extract is made as above and acidified with 0.2 per cent hydrochloric acid, the figures are the same except in the case of nitrogen which is nearly 1.0 per cent lower. When an extract made by means of distilled water is saturated with carbon dioxide the figures are the same as those obtained from dialysis. Further work is in progress.

THE DISTRIBUTION OF QUERCIMERITRIN IN THE COTTON PLANT (GOSSYPIUM HERBACEUM).[8]

By ARNO VIEHOEVER, LEWIS H. CHERNOFF, and CARL O. JOHNS.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

Quercimeritrin has hitherto been isolated only from the petals of the cotton plant. The authors have found that it also occurs in the calyx and leaves of the cotton plant. The quercimeritrin was isolated by the method of Perkins. It was identified by its melting point and by the fact that it gave quercitin on hydrolysis.

CEDRIN, A GLUCOSIDE FROM THE SEEDS OF SIMABA CEDRON.[8]

By ARNO VIEHOEVER, G. A. GEIGER and CARL O. JOHNS.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

Cedrin, the bitter principle of *Simaba Cedron*, has been used in medicine for some time, but seems to have received little attention from a chemical point of view. The authors obtained it by extracting the seeds of *Simaba Cedron* with 95 per cent alcohol. It crystallized in truncated rhombohedrons which melted with

decomposition at 265°C. Analysis gave carbon 61.74 per cent, hydrogen 6.46 per cent. It failed to give a test for nitrogen. The molecular weight in chloroform was 406. These figures agree well with $C_{21}H_{26}O_8$. On hydrolysis cedrin gave a sugar which has not been identified. Solubility determinations of cedrin were made.

A SAPONIN FROM YUCCA ANGUSTIFOLIA.[8]

BY ARNO VIEHOEVER, LEWIS H. CHERNOFF, AND CARL O. JOHNS.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

A saponin was isolated from the root stock of *Yucca angustifolia*. This gave a molecular weight in phenol of 773. It contained 52.97 per cent carbon, 6.77 per cent of hydrogen, and 40.26 per cent of oxygen. These figures agree closely with $C_{36}H_{56}O_{20}$, which is suggested as a provisional formula. On hydrolysis a sugar was obtained which gave the reactions characteristic of galactose.

A SAPONIN FROM YUCCA RADIOSA.[8]

BY CARL O. JOHNS, G. A. GEIGER, AND ARNO VIEHOEVER.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

A saponin has been isolated from *Yucca radiosa* by extracting the ground stem with alcohol. In phenol it gave a molecular weight of 804. Analysis gave carbon 54.01 per cent, hydrogen 7.06 per cent, and oxygen 38.93 per cent. These figures agree with $C_{37}H_{58}O_{20}$, which is suggested as a provisional formula. On hydrolysis a sugar was obtained. Reactions indicate that this is glucose or mannose. Attempts will be made to crystallize this sugar.

INVESTIGATION OF THE KJELDAHL METHOD FOR DETERMINING NITROGEN.[8]

By I. K. PHELPS and H. W. DAUDT.

A study of various conditions of digestion of the Kjeldahl-Gunning-Arnold method was made on pyridine zinc chloride. It was found that mercuric oxide could not be replaced by various other catalysts and that the use of sodium sulfate in the place of potassium sulfate gave unreliable results. Experiments made with specially constructed lead condensers, using varying proportions of potassium sulfate and sulfuric acid showed complete decomposition of the pyridine when 0.3 gm. of pyridine zinc chloride was digested for $2\frac{1}{2}$ hours in an open flask with a boiling mixture of 25 cc. of concentrated sulfuric acid, 10 gm. of potassium sulfate, and 0.7 gm. of mercuric oxide. The same proportions of reagents under the same conditions were found to give reliable results of the quantity of nitrogen present in these classes of cyclic compounds: pyrrole, pyrrolidine, pyridine, piperidine, quinoline, isoquinoline, purine, imidazole, quinoxaline, and quinazolone. Incomplete decomposition was found to result in the case of pyridine when the proportion of potassium sulfate was not sufficiently high.

THE NITROGEN DISTRIBUTION IN JACK BEANS, COW-PEAS, AND WHEAT.[8]

By J. F. BREWSTER.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

The nitrogen distribution in jack beans, cow-peas, and wheat were determined by the Van Slyke method.

A NEW SUGAR FROM THE AVOCADO (MANNOKETOHEPTOSE).[8]

By F. B. LA FORGE.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

The substance occurs free in the pulp of the ripe fruit, from which it was obtained by extraction with water. The extract was concentrated and freed from gum by precipitation with

alcohol. An heptatomic alcohol (perseit) crystallized from the concentrated alcoholic solution, leaving the sugar. From the syrup obtained by evaporation, the bromophenylhydrazone was prepared (m. p. 178°). The analysis of this as well as that of the osazone (m. p. 200°) shows the sugar to be a heptose. Cleavage of the bromophenylhydrazone with benzaldehyde yielded the free sugar which crystallized from dilute alcohol in six-sided prisms (m. p. 152°, $[\alpha]^{20}_{D} = +29.5$). The sugar is non-fermentable and the fact that bromine is without oxidizing effect indicates that it is a ketose. The osazone appears to be identical with that of mannoheptose.

THE FORMATION AND STRUCTURE OF THE FIBRIN GEL.[10]

By W. H. HOWELL.

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FURTHER INVESTIGATIONS ON OVER-ACTIVITY OF THE CERVICAL SYMPATHETIC NERVE.[10]

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By OTTO FOLIN and R. D. BELL.

With the Assistance of G. Le B. Foster.

THE CONTINUOUS INSUFFLATION THROUGH THE HUMERUS IN THE FOWL.[11]

By A. L. MEYER and S. J. MELTZER.

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[10] Presented from the Physiological Society in joint session of the Federation.

[11] Presented from the Pharmacological Society in joint session of the Federation.

THE INFLUENCE OF THE ADRENALS ON THE KIDNEY.[11]

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STUDIES IN EXPERIMENTAL DIABETES AFTER PANCREATECTOMY.[1]

By ALBERT A. EPSTEIN and GEORGE BAEHR.

(*From the Pathological Laboratory of the Mount Sinai Hospital, New York.*)

(Received for publication, November 11, 1915.)

Frequent estimation of the blood sugar at short intervals has only been possible since the recent introduction of so called microchemical methods. Undoubtedly the greatest field of usefulness for these methods is in experimental work on laboratory animals, for they largely eliminate the possibility of a psychic hyperglycemia and of the hyperglycemia following more extensive bleeding. In dogs and cats they also obviate the necessity of obtaining blood from the larger vessels with consequent increase in the blood sugar due to the operative procedures. The advantages gained more than counterbalance the slightly larger limits of technical error that are likely to occur from the use of small quantities of blood.

In the following experiments, a modification of the excellent picric acid method of Lewis and Benedict was used. By means of a technique described recently by one of us,[2] we have been able to obtain remarkably accurate determinations on 0.1 cc. and 0.2 cc. of blood instead of the 2 cc. originally recommended. The method is superior to that of Bang both in simplicity and in accuracy (Epstein[3]).

The frequently repeated estimation of the sugar in such small quantities of blood enabled us to follow the entire course of the hyperglycemia which develops after a pancreatectomy, much more minutely than was previously possible. For this purpose cats were found to be especially suitable, because of the ease with

[1] This work was carried out under the tenure of the George Blumenthal, Jr., and the Moses Heineman Fellowships.

[2] Epstein, A. A., *Jour. Am. Med. Assn.*, 1914, lxiii, 1667.

[3] Epstein, A. A., *Jour. Am. Med. Assn.*, 1915, lxv, 1748.

which sufficient blood can be obtained from an ear vein by puncture with a needle, without disturbing the animal.

As originally pointed out by Minkowski,[4] the technique of totally extirpating the pancreas in the cat is more difficult than in the dog, chiefly because of the close relation of one part of the organ to the portal vein. But after a brief experience the ability to do a total extirpation without damage to the portal vein or interference with the blood supply of the duodenum was readily acquired. Post mortem, any tissue near the original site of the organ which even remotely resembled pancreatic tissue was subjected to microscopic examination. In none of the experiments which form the basis of this communication were any fragments of pancreas ever left behind, nor did necrosis of the duodenum ever occur.

In our experience the only disadvantage of using cats is that they succumb sooner than dogs after total pancreatectomy. They are, therefore, less suitable for prolonged metabolic study. But for the purpose of the present investigation, this does not constitute a disadvantage.

After pancreatectomy the animals usually are very thirsty and drink water in large amounts, although they may refuse to eat. This fact necessitated our obtaining some knowledge concerning possible variations in blood volume which might affect the sugar concentration of the blood. For it appeared conceivable that the ingestion of rather large amounts of fluid might cause a dilution of the blood and thus mask an actual increase in its constituents, including sugar. This obviously important point has been universally overlooked by workers in this field. It has been discussed by us in a recent paper dealing with the relation of the percentile to the absolute hyperglycemia.[5]

Changes in the blood volume can be ascertained by means of repeated determinations of the relative amounts of plasma and cells present in the blood. Simultaneously with each sugar determination an estimation of the proportion of plasma and blood cells was therefore always made (by means of a simplified hematocrit) and changes in the blood volume were calculated. It can be safely assumed, for reasons which need not be stated here, that

[4] Minkowski, O., Untersuchungen über den Diabetes mellitus nach Extirpation des Pankreas, Leipsic, 1893, 77.

[5] Epstein, A. A., and Baehr, G., *Jour. Biol. Chem.*, 1914, xviii, 21.

the actual number of cells in the blood stream remains practically constant from hour to hour or even from day to day. A decrease in the proportion of blood cells in a unit of blood therefore signifies an addition to the fluid portion of the blood and hence an increase in the total volume. If the proportion of blood cells found at the beginning of the experiment be taken as the standard for comparison, the subsequent increase or decrease in the blood volume can be ascertained by dividing the percentage of blood cells in a given determination into the percentage found at the outset.

In the following tables Column 2 contains the percentage of plasma, Column 3 the percentage of blood cells, and Column 4 the relative blood volume calculated from the figures in Column 3.

In view of the changes which take place in the blood volume after pancreatectomy, the percentage of sugar found at each determination must therefore be proportionally magnified or decreased, if we are to obtain a correct idea of the actual rate of increase in the sugar content. Column 6 therefore represents the percentage of sugar which would have been found had the blood volume remained as it was before the experiment. This gives us a new and surprising insight into the actual increase in the amount of sugar in the circulating blood.

TABLE I.

Cat 9.

Weight 3,750 Gm. Complete Pancreatectomy.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	64.3	35.7	100.0	0.084	0.084
After "	65.2	34.8	102.6	0.240	0.266
16	72.1	27.9	127.8	0.256	0.327
21	69.2	30.8	115.8	0.264	0.306
26	73.5	26.5	134.7	0.320	0.431
42	73.4	26.6	134.2	0.320	0.435
52	76.3	23.7	150.6	0.280	0.422
70	74.7	25.3	141.1	0.290	0.409
96	72.1	27.9	127.9	0.372	0.476
100	75.6	24.4	146.3	0.412	0.603
119	73.1	26.9	132.5	0.560	0.742
124	73.4	26.6	134.2	0.580	0.778
141	73.6	26.4	135.2	0.620	0.838

Total glycogen.

	gm.	per cent
Liver	0.184	0.184
Muscle	——	0.224

Incoagulable N in Blood.

Found.	Corrected.
per cent	*per cent*
0.240	0.324

The blood volume of Cat 9 (Table I) increased after the removal of the pancreas, reaching 134.7 per cent after 24 hours. Subsequently the volume fluctuated above and below this figure, depending on the ingestion of water. The highest value attained by the blood volume was 150.6 per cent 52 hours after the pancreatectomy.

Coincident with the increase in the blood volume the blood sugar content rose gradually from 0.084 per cent before the operation to 0.431 per cent 26 hours after. It then remained at about the same level until 100 hours after the removal of the pancreas when a sudden rise to 0.603 per cent occurred. From that time on, the increase was progressive, reaching 0.838 per cent after 141 hours. The animal which by this time was markedly emaciated became stuporous. The experiment was therefore discontinued; the animal was killed and the liver, muscle, and blood were subjected to further analysis.

This terminal rise in the percentage of blood sugar was observed in three other cats on which similar studies were made after pancreatectomy. (See protocols at end of paper.) In fact the characteristically progressive rise in the blood sugar presaged the death of the animals during the following 24 to 48 hours. This sudden terminal rise appeared, however, at least 24 to 36 hours before the onset of stupor.

As this change in the blood sugar is a terminal phenomenon, one might at first suppose that it represents the result of tissue decomposition with increased sugar production. But the rise always appeared when the animals were still in good condition and quite lively. Furthermore, a simultaneous diminution in the glycosuria demonstrated that the rapidly progressing hyperglycemia was the result of a sugar retention in the blood due to an impaired function of the kidneys.

TABLE II.

Course of Glycosuria in Cat 9.

Time after operation.		Quantity of urine.	Total sugar.		Acetone.	Albumin
hrs.	*hrs.*	*cc.*	*per cent*	*gm.*		
1– 24...	24	125.0	12.5	15.6	0	0
24– 48...	24	252.0	7.3	18.4	+++	0
48– 66...	18	200.0	5.7	11.4	++	+
66– 84...	18	238.0	2.0	4.8	+	+
84–108...	24	202.0	0.6	1.2	0	++
108–141...	33	45.0	+	+	0	++

Table II shows that after 84 hours only traces of sugar appeared in the urine, and that after 48 hours albumin and casts were present in increasing amounts. Coincident with the appearance of the marked albuminuria, the hyperglycemia increased, whereas the glycosuria and acetonuria, which had previously been very marked, progressively diminished. The urine contained no acetone after the 84th hour and only traces of sugar after the 108th hour. In view of the progressively increasing hyperglycemia at this time, the diminution in the excretion of sugar can only be the result of a functional insufficiency of the kidney. Similarly the stupor which began during the last 12 hours of the experiment was probably due to the retention of the acetone bodies, also resulting from the renal insufficiency.

It is interesting to note, however, that the progressive increase in the blood sugar began long before the onset of stupor. As seen in the table, the marked diuresis which began shortly after the operation, persisted for at least 48 hours after the excretion of sugar and acetone began to fail. This fact may be interpreted as an indication that the impairment of function which affected the elimination of sugar and acetone was not of circulatory origin.

The terminal hyperglycemia observed in the cat experiments has its analogue in human diabetes. Whenever exceptionally high sugar values were reported in the literature, we have found that the estimations were made within a day or two of death (Naunyn,[6] 0.7 per cent, Lépine,[7] 1.06 per cent, von Noorden,[8] 0.85 per cent

[6] Naunyn, B., Der Diabetes mellitus, Vienna, 2nd edition, 1906, 189.

[7] Lépine, R., *Rev. de méd.*, 1897, xvii, 832.

[8] Von Noorden, C., Handb. d. Pathologie des Stoffwechsels, Berlin, 2nd edition, 1906, ii, 3.

and 1.01 per cent, etc.). In one case of von Noorden's in which the blood sugar was 1.01 per cent the urine contained only traces of sugar. Post mortem, no nephritis was discoverable. From our experimental observations, it is apparent that all these high figures in human diabetes are probably also due to the retention of sugar resulting from a renal insufficiency. From the experimental evidence it would seem that a progressively increasing percentage of sugar in the blood is an indication of impending death. In a patient with diabetes whom we had the opportunity of observing recently, we found conditions similar to those observed experimentally. In this case as in the experimental animals, a progressive increase in the blood sugar preceded the onset of coma.

In view of the above phenomena it was thought to be of interest to reproduce an absolute renal insufficiency in depancreatized animals, in order to ascertain its effect upon the sugar content of the blood. A total extirpation of the pancreas followed by double nephrectomy was carried out upon six cats, of which three were well nourished and the others had been starved for 9 days.

Table III (Cat 10) is an example of the observation made on the group of well nourished animals.

TABLE III.

Cat 10.

Weight 3,200 Gm. Well Nourished. Pancreatectomy and Double Nephrectomy.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.....	64.6	37.4	100.0	0.085	0.085
After "	63.7	36.7	97.2	0.206	0.200
14.....	75.7	24.3	145.7	0.640	0.932
19.....	74.7	25.3	140.0	0.816	1.142
24.....	75.4	24.6	144.0	1.050	1.512
40.....	77.7	22.3	158.6	1.080	1.713

Total glycogen.

	gm.	*per cent*
Liver.....	0.278	0.42
Muscle.....	——	0.99

Incoagulable N in blood.

Found.	Corrected.
per cent	*per cent*
0.280	0.444

Cat 13. Control.

Well nourished. Not operated upon.

Total glycogen.

	gm.	*per cent*
Liver	11.951	14.40
Muscle	———	1.70

Incoagulable N in blood.

Found.	Corrected.
per cent	*per cent*
0.073	0.073

The figures obtained are very striking. After the operation the blood sugar rose very rapidly. The animal meanwhile exhibited thirst, although it took no food. As both kidneys had been removed, the ingestion of fluid resulted in a simultaneous increase in the blood volume. After 40 hours the blood volume had increased more than 58 per cent. This big factor, as previously stated, must be considered if we are to obtain a correct idea of the actual accumulation of the sugar in the blood. The percentage of sugar found at each determination was therefore proportionately magnified as seen in Column 6. During the course of the experiment the blood sugar thus rose from 0.085 to 1.713 per cent; in other words, it increased more than twenty-fold.

TABLE IV.

Cat 20.

Fasted 9 Days before Operation. Initial Weight 3,300 Gm. Final Weight 2,880 Gm. Pancreatectomy and Double Nephrectomy.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	62.3	37.7	100.0	0.058	0.058
After "	58.0	42.0	90.0	0.100	0.090
½	56.7	43.3	87.8	0.120	0.104
7	65.2	34.8	108.3	0.272	0.295
18	70.0	30.0	126.0	0.384	0.484
23	74.2	25.8	146.1	0.408	0.596
27	74.5	25.5	146.0	0.500	0.730
30	76.0	24.0	157.0	0.568	0.892
41	76.0	24.0	157.0	0.740	1.161
46	77.2	22.8	165.1	0.998	1.647
48	77.1	22.9	165.0	1.120	1.980

Total glycogen.

	gm.	per cent
Liver	0	0
Muscle	—	0.06

Incoagulable N in blood.

Found.	Corrected.
per cent	*per cent*
0.241	0.398

Cat 22. Control.

Fasted, but not operated upon.

Total glycogen.

	gm.	per cent
Liver	0.014	0.030
Muscle	——	0.560

Incoagulable N in blood.

Found.	Corrected.
per cent	*per cent*
0.046	0.046

The experiment on Cat 20 (Table IV) serves to illustrate the effect of a similar procedure upon an animal that had fasted for 9 days. In this instance the blood volume gradually increased until at the end of 48 hours it was 65 per cent larger than at the beginning of the experiment. Correcting the sugar values obtained for this dilution, the sugar in the blood is seen to have risen from 0.058 to 1.980 per cent. In other words, at the end of 48 hours the blood contained thirty-four times as much sugar as before the experiment.

The rate of increase in the sugar content of the blood is different in the two sets of experiments presented. In the early stages of the experiment, the hyperglycemia developed much more rapidly in the well nourished than in the starved animals. The difference is best seen by comparing the curves of the results obtained on Cat 10, which was well nourished, with that of Cat 20, which was starved.

The blood sugar of Cat 10 is as high after 19 hours as that of Cat 20 after 40 hours. The very rapid increase in the hyperglycemia in Cat 10 continued for the first 24 hours, but proceeded rather slowly after that. The progress of the hyperglycemia in Cat 20, on the other hand, was much slower than in Cat 10 for

the first 41 hours; after this the rise in the sugar content was very rapid.

The difference in the rate of increase in the hyperglycemia in the two sets of animals can be accounted for by a difference in the reserve of carbohydrate. A control to Cat 10 (Cat 13) which was not operated upon, showed 14.4 per cent of glycogen in the liver and 1.7 per cent in the muscles. (Glycogen was determined

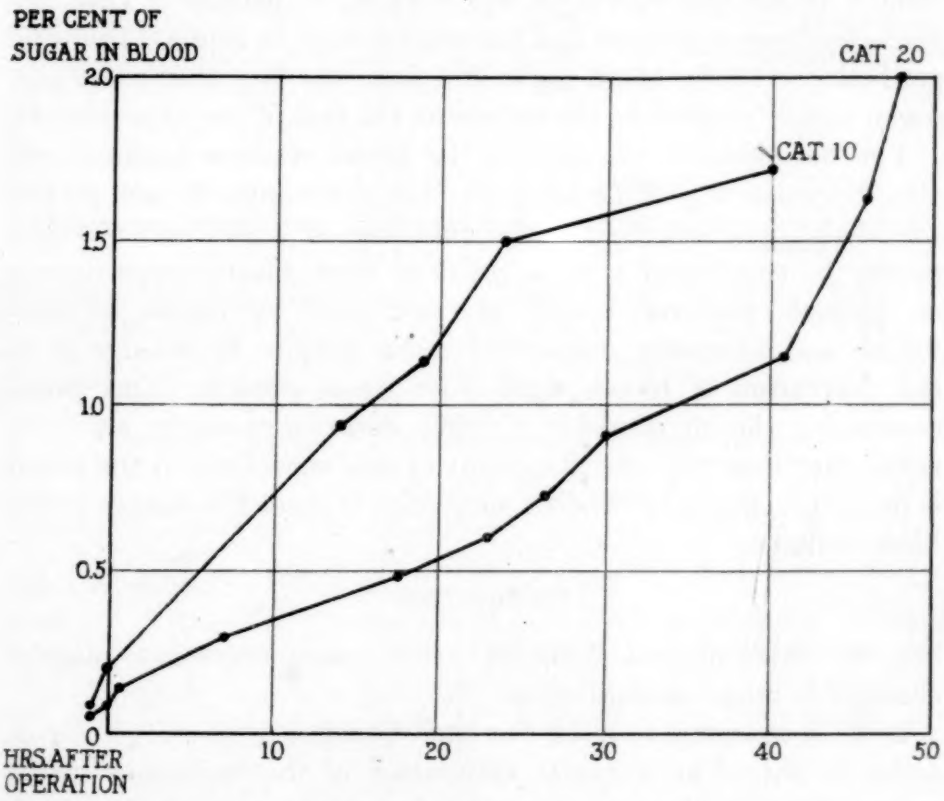

FIG. 1. Curves illustrating rate of increase in blood sugar after pancreatectomy and double nephrectomy.

Cat 10 well nourished; Cat 20 fasted.

by Pflüger's method.) The liver of the control animal to Cat 20 (Cat 22), which had also been starved for 9 days, but had not been operated upon, contained only 0.03 per cent of glycogen, and the muscles 0.56 per cent. On the other hand, the livers and muscles of Cats 10 and 20 yielded the following results:

Cat 10.		*Cat 20.*	
Liver.....	0.42 per cent glycogen.	Liver.....	0 per cent glycogen.
Muscle.....	0.99 " "	Muscle.....	0.06 " "

Evidently the extraordinary accumulation of sugar in the blood does not hinder the progressive decomposition of glycogen. In both animals the accumulation of sugar in the blood can, at least in part, be accounted for by a mobilization of the reserve carbohydrate from the liver and muscles. That this is true is further evidenced by the much more rapid accumulation of sugar in the blood of the well nourished animal (Cat 10) in which the carbohydrate reserve before the operation was very much greater. That the glycogenolysis is gradual and not sudden may be implied from the gradual rise in the blood sugar and from the fact that some glycogen is still present in the organs at the end of the experiments.

The incoagulable nitrogen in the blood of these animals was also determined (Folin's method), but the results do not permit any deductions concerning the existence of a dextrose-nitrogen ration in the blood. It is possible that similar experiments on animals rendered totally glycogen-free, by means of starvation and adrenalin, may yield more definite knowledge as to the derivation of blood sugar from tissue protein. One point concerning the incoagulable nitrogen deserves mention, and that is the fact that the actual amount of this substance in the blood is found much greater when a correction is made for change in the blood volume.

CONCLUSIONS.

1. In experimental diabetes after pancreatectomy marked changes in blood volume occur.

2. It is essential to allow for such changes in blood volume in order to obtain an accurate estimation of the variations in the sugar, incoagulable nitrogen, and other constituents of the blood.

3. The hyperglycemia in experimental diabetes after pancreatectomy in the cat mounts progressively in the terminal stages of the condition because of diminution in the permeability of the kidneys.

4. This furnishes an explanation of the excessive rise in the blood sugar of diabetics prior to the development of coma.

5. Experimental interference (by double nephrectomy) with the glycosuria of depancreatized animals also causes a rapidly progressing hyperglycemia.

6. This accumulation of sugar in the blood is largely due to the gradual mobilization of carbohydrate from the liver and muscles.

TABLE V.

Cat 2.

Weight 2,720 Gm. Pancreatectomy on Normal Animal.

Time.	Blood sugar.	
hrs.	*per cent*	
Before operation	0.079	
After "	0.266	
15	0.250	
24	0.292	Relative blood volume not determined in this animal.
36	0.212	
44	0.238	
60	0.208	
72	0.246	
84	0.244	
108	0.456	
111¼	0.512 (killed.)	

	Weight. *gm.*	Glycogen. *gm.*	*per cent*
Liver	74	0.213	0.3
Muscle	44	0.326	0.74

TABLE VI.

Cat 3.

Weight 3,190 Gm. Pancreatectomy on Normal Animal.

Time.	Blood sugar.	
hrs.	*per cent*	
Before operation	0.090	
After "	0.272	Relative blood volume not determined in this animal.
12	0.286	
36	0.272	
64	0.290	
89	0.380	
100	Died.	

TABLE VII.

Cat 32.

Weight 1,900 Gm. Pancreatectomy. Starved 10 Days.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	56.3	43.7	100.0	0.058	0.058
After "	58.1	41.9	104.3	0.106	0.110
43	61.8	38.2	114.4	0.193	0.221

Animal died subsequently; no further analyses were made.

TABLE VIII.

Cat 31.

Weight 2,300 Gm. Pancreatectomy. Starved 10 Days.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	55.2	44.8	100.0	0.055	0.055
After "	48.6	51.4	81.1	0.107	0 093
20	54.1	45.9	97.9	0.226	0.221
86	50.6	49.4	90.7	0.760	0 689

TABLE IX.

Cat 5.

Weight 2,400 Gm. Nephrectomy on Normal Cat.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	63.5	36.5	100.0	0.088	0.088
After "	59.1	40.9	89.2	0.254	0.227
14	63.9	36.1	101.1	0.140	0.142
24	64.0	36.0	101.4	0.150	0.152
40	68.5	31.5	115.9	0.122	0.141
48	68.2	31.8	114.8	0.109	0.125
63	68.0	32.0	114.1	0.140	0.160
88	65.2	34.8	104.9	0.148	0.155
88½	Died.				

TABLE X.

Cat 6.

Weight, 2,800 Gm. Nephrectomy on Normal Animal.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	68.1	31.9	100.0	0.079	0.079
After "	70.4	29.6	107.9	0.308	0.332
18	71.9	28.1	113.5	0.120	0.136
43	78.8	21.2	150.5	0.180	0 271

TABLE XI.

Cat 8.

Nephrectomy on Normal Animal.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	57.9	42.1	100.0	0.079	0.079
After "	60.0	40.0	105.2	0.360	0.379
17	72.7	27.3	154.2	0.140	0.216
24	73.0	27.0	155.9	0.142	0.221

TABLE XII.

Cat 21.

Weight 3,100 Gm. Nephrectomy. Starvation and Phlorhizin. Starved 5 Days. 2 Days Phlorhizin (0.5 Gm.). Weight before Operation 2,680 Gm.; at Death 2,550 Gm.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	60.9	39.1	100.0	0.042	0.042
After "	50.0	50.0	78.2	0.077	0.060
6	Not taken.			0.196	
16	64.0	36.0	108.6	0.080	0.087
$21\frac{3}{4}$	60.6	39.4	99.2	0.070	0.069
$24\frac{1}{2}$	61.1	38.9	100.5	0.076	0.076
29	61.9	38.1	102.6	0.078	0.080
40	62.8	37.2	105.1	0.090	0.095
45	62.0	38.0	102.9	0.117	0.120
47	61.1	38.9	100.5	0.125	0.126

Killed.

	Weight.	Glycogen.
	gm.	*gm.*
Liver	100	3.717
Muscle	87.5	0.152
Incoagulable N in blood	0.281 gm. per 100 cc.	

TABLE XIII.

Cat 24.

Nephrectomy. Starved 9 Days. Phlorhizin Twice (0.5 Gm.).

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.....	44.4	55.6	100.0	0.057	0.057
After operation (5 min.).	61.0	39.0	142.6	0.102	0.145
6.....	39.4	60.6	91.7	0.081	0.074
20.....	44.7	55.3	100.5	0.085	0.085
25.....	43.5	56.5	98.8	0.075	0.074
30.....	50.9	49.1	113.2	0.060	0.068

Died during next 6 hours.

TABLE XIV.

Cat 26.

Nephrectomy. Starvation and Phlorhizin. Fasted 9 Days. Phlorhizin Twice (0.5 Gm.). Weight before Killing 2,300 Gm.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.....	61.4	38.6	100.0	0.052	0.052
After "	57.0	43.0	89.8	0.078	0.070
13.....	61.7	38.3	100.8	0.080	0.081
23.....	59.6?	40.4	95.5	0.064	0.061

Killed.

	Weight. *gm.*	Glycogen. *gm*
Liver.....	81	1.362
Muscle.....	74	0.055

Incoagulable N in blood (15 cc.).....0.136 gm. per 100 cc.

TABLE XV.

Cat 12.

Weight 2,830 Gm. Nephrectomy and Pancreatectomy on Normal Animal.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.....	64.6	35.4	100.0	0.090	0.090
After "	57.05	42.95	82.3	0.210	0.173
17½.....	73.2	26.8	132.0	0.480	0.633
21¾.....	75.6	24.4	145.1	0.720	1.044
22½.....	77.0	23.0	154.0	0.930	1.432

TABLE XVI.

Cat 4.

Nephrectomy and Pancreatectomy on Normal Animal.

Time.	Blood sugar.	
	per cent	
Before operation	0.089	
After onset of anesthesia	0.099	Relative blood volume not determined in this animal.
10 min. after pancreatectomy	0.206	
40 " " "	0.224	
(10 min. after nephrectomy.)		
8½ hours after pancreatectomy	0.392	
16 " " "	0.540	
20¼ " " "	0.700	
25 " " "	0.900	

TABLE XVII.

Cat 23.

Weight 2,200 Gm. Pancreatectomy and Nephrectomy. Fasted 9 Days before Operation. Phlorhizin.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	57.5	42.5	100.0	0.050	0.050
During "	53.0	47.0	90.4	0.062	0.056
After "	54.2	45.8	93.0	0.095	0.088
6½	59.2	40.8	104.1	0.210	0.219
20	68.5	32.5	130.8	0.618	0.808
24½	73.1	26.9	158.0	0.680	1.074
30	73.7	26.3	161.6	0.690	1.115

Cat died subsequently; other analyses not made.

TABLE XVIII.

Cat 25.

Weight 2,860 Gm. Pancreatectomy and Nephrectomy. Fasted 10 Days. Phlorhizin (0.5) Gm. on 5th and 6th Days.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	48.7	51.3	100.0	0.050	0.050
After "	39.5	60.5	84.8	0.064	0.054
14	51.0	49.0	104.7	0.260	0.272
21	57.0	43.0	119.3	0.456	0.544
23	58.8	41.2	124.5	0.416	0.518

	Weight. gm.	Glycogen. gm.
Liver	90	0.014
Muscle	82	0.074

Incoagulable N in blood.....0.145 gm. per 100 cc.
Corrected.....0.180 " " 100 "

TABLE XIX.

Cat 29.

Initial Weight, 3,950 Gm.; Final, 2,920 Gm. Nephrectomy. Starvation and Phlorhizin.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.....	48.2	51.8	100.0	0.059	0.059
After "	43.3	56.7	91.4	0.130	0.119
43.....	48.4	51.6	100.5	0.088	0.088

Killed.

	Weight. gm.	Glycogen. gm.
Liver	72	0.748
Muscle	65	0.126

Incoagulable N in blood.....0.183 gm. per 100 cc.

TABLE XX.

Cat 30.

Initial Weight, 3,500 Gm.; Final, 2,100 Gm.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.....	54.5	45.5	100.0	0.057	0.057
After "	53.3	46.7	97.4	0.154	0.150
43.....	58.5	41.5	109.6	0.072	0.079

	Weight. gm.	Glycogen. gm.
Liver	95.5	2.196
Muscle	62.5	0.167

Incoagulable N in blood.....0.284 gm. per 100 cc.

THE EFFECT OF PHLORHIZIN ON THE FORMATION OF GLYCOGEN IN THE LIVER.[1]

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Although it is now generally recognized that the glycosuria of phlorhizin poisoning is due to a direct action on the kidney, some authors (Grube,[2] Biedl and Kolisch,[3] Gigon[4]) still believe that phlorhizin may also possess a specific effect upon the liver. The following observations are presented because they conclusively controvert this possibility of a direct mobilization of liver glycogen by phlorhizin. In fact they seem to indicate an interesting condition which has not previously been surmised, that in the absence of the kidneys the presence of phlorhizin in the body actually stimulates the accumulation of glycogen in the liver.

Many of the following experiments were carried out for another purpose and formed the basis of the previous communication upon some "Studies in Experimental Diabetes after Pancreatectomy." They may be classified into seven series as seen in the following tables. In all the experiments cats weighing 2,500 to 3,000 gm. were used. Phlorhizin was administered subcutaneously, 0.5 gm. in 5 cc. of olive oil, on 2 successive days.

The glycogen was determined by Pflüger's method. The blood sugar was determined by means of the microchemical method described by one of the authors.[5]

The experiments tabulated in Tables I, II, and III demonstrate the result of starvation and of starvation plus phlorhizin upon the

[1] This work was carried out under the tenure of the George Blumenthal, Jr., and the Moses Heineman Fellowships.

[2] Grube, K., *Arch. f. d. ges. Physiol.*, 1909, cxxviii, 118.

[3] Biedl, A., and Kolisch, R., *Verhandl. d. Cong. f. inn. Med.*, 1900, xviii, 573.

[4] Gigon, A., *Ztschr. f. klin. Med.*, 1907, lxiii, 420.

[5] Epstein, A. A., *Jour. Am. Med. Assn.*, 1914, lxiii, 1667.

glycogen content of the liver and muscles. The most marked effect is exerted upon the liver, the glycogen in five of the seven animals being reduced to little more than a trace, and completely disappearing in two others that received phlorhizin.

The experiments in Table V seem to indicate that bilateral nephrectomy also results in a mobilization of carbohydrate from the liver and to a less extent from the muscles. This is evidenced by the disappearance of most of the glycogen from the

TABLE I.

Normal Animal.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
83	11.951	14.4	88	1.460	1.66	0.078

TABLE II.

Starved for 5 to 9 Days.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
40	0.008	0.02	55.0	0.063	0.11	0.079
47	0.014	0.03	33.5	0.188	0.56	
45.1	0.025	0.06	41.3	0.021	0.05	
31.0	0.013	0.04	32.0	0.005	0.02	
Average		0.04			0.19	

TABLE III.

Starved and Phlorhizinized for 9 Days.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
68	0.204	0.30	53.5	0.148	0.28	0.065
98			74.0	0.170	0.23	0.064
48.5			47.5	0.118	0.25	0.047
Average					0.25	

TABLE IV.

Starved and Phlorhizinized for 9 Days before Operation. Then Double Nephrectomy. Starved 3 More Days and Killed.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar.	
						Before operation.	Before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
100	3.717	3.72	87.5	0.152	0.17	0.042	
81	1.362	1.68	74.0	0.055	0.07	0.052	
72	0.748	1.04	65.0	0.126	0.20	0.059	0.088
95.5	2.196	2.29	62.5	0.167	0.27	0.057	0.079
Average		2.18			0.18		

TABLE V.

Animals 3 Days after Double Nephrectomy. Starved during the 3 Days. Well Fed before Operation.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar.	
						Before operation.	Before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
61	0.880	1.28				0.088	0.155
75	0.278	0.37				0.079	0.150
75	0.044	0.05	81	0.593	0.73	0.079	0.156
Average		0.57					

TABLE VI.

Pancreatectomy and Double Nephrectomy. Well Fed before Operation. Killed 2 Days after Operation.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
66	0.278	0.42	85	0.845	0.99	0.085
114	0.750	0.66	90	2.850	3.17	0.090
Average		0.54				

TABLE VII.

Starved and Phlorhizinized for 9 Days. Pancreatectomy and Double Nephrectomy. Killed 2 Days after Operation.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
79			103.5	0.064	0.06	0.058
90	0.014	0.02	82.0	0.074	0.09	0.050
Average					0.07	

liver of cats which were previously well fed and by the hyperglycemia which was present after the operation.

Pancreatectomy and double nephrectomy upon previously well fed cats resulted in a rapid diminution though not a complete disappearance of the liver glycogen in 48 hours (Table VI). 48 hours after a similar operation upon cats which were previously phlorhizinized and starved for 9 days (Table VII) the liver was found to be practically glycogen-free.

Important and interesting results were obtained in the four experiments listed in Table IV. These animals were phlorhizinized and starved for 9 days and then a double nephrectomy was performed. After 3 more days of starvation they were killed. Before the operation there was a fairly marked hypoglycemia because of the long continued phlorhizin glycosuria. After the nephrectomy the percentage of blood sugar rose to the normal figure. The analyses of the muscles demonstrated the presence of glycogen in amounts similar to that found in animals starved, or starved and phlorhizinized for a similar length of time but in which the kidneys had not been removed (Tables II and III).

The results of the analyses of the livers (Table IV) were, however, very striking. From the results obtained in the seven experiments of Tables II and III it was obvious that at the time of the operation the livers of all four animals must have contained little or no glycogen. And yet 3 days subsequent to the operation considerable quantities of glycogen were present in the liver although the animals had continued to starve. Starvation and phlorhizin for this length of time ordinarily free the liver of its glycogen (Tables II and III). The operation of double

nephrectomy in a normal animal also induces a rapid diminution of the liver glycogen.

There are only two possible explanations to account for the reaccumulation of glycogen in the liver of phlorhizinized animals after nephrectomy. One is that because of a possible disturbance in carbohydrate metabolism produced by the phlorhizin, an excess of sugar accumulates in the body after the nephrectomy. This explanation is rendered improbable by the fact that the percentage of blood sugar in these animals never rose above the normal figure.

The only possible explanation therefore appears to be that phlorhizin exerts a specific effect upon the liver which does not become evident unless the kidneys are removed. In other words, phlorhizin in some way stimulates the liver to build up glycogen. With the kidneys intact, the glycosuric action of phlorhizin is so much more powerful than this liver action that the glycogen accumulations in the liver are rapidly diminished. Upon removal of the kidneys, however, the glycosuric action is eliminated and the effect upon the liver becomes manifest in a rapid building up of glycogen, even in a state of extreme starvation.

THE USE OF PHLORHIZINIZED DOGS TO DETERMINE THE UTILIZABLE CARBOHYDRATE IN FOODS.

THE FOOD VALUE OF COMMERCIAL GLUCOSE.

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The sum total of *d*-glucose which a given food may introduce into the metabolism will depend (1) upon its content of free glucose and of polysaccharides that yield glucose by digestion; and (2) upon its content of other substances which, with or without digestion, are capable of transformation into *d*-glucose within the body; for example, other sugars besides glucose, polysaccharides whose digestion yields other sugars besides glucose, and proteins and protein digestion products. While in many cases ordinary methods of proximate food analysis may enable us to estimate the glucose equivalents of foods with sufficient accuracy for clinical purposes, this is not true for all foods. The soluble fraction of the nitrogen-free extract may contain a mixture of sugars incapable of separation or exact measurement, the insoluble portion may not be sharply separable into the utilizable and non-utilizable fractions, etc.

The idea suggested itself of passing a food through the body of a completely phlorhizinized dog and measuring its total yield of extra sugar in the urine, the well known phlorhizin technique being thus applied to a problem of food analysis. The extra sugar recovered would then naturally represent nothing which was either indigestible, unabsorbable, or unassimilable. If the food contained protein, such sugar as came from this source could be reckoned from the urinary nitrogen and would not be confused with extra sugar.

On previous occasions, for purposes of ascertaining directly their value in diabetic management, we have determined the

total glucose equivalents of breads by metabolism experiments with completely phlorhizinized dogs and obtained results which were in close harmony with the values calculated from the proximate food analysis. The present paper describes an attempt to utilize the same method to determine directly what percentage of commercial glucose, when fed by mouth, is capable of ultimate absorption and assimilation as *d*-glucose. Five experiments were performed, in each of which the dogs received a weighed dose of commercial glucose by mouth, and a control dose of a corresponding weight of chemically pure *d*-glucose. The results are given in Table I.

TABLE I.

Results of the Administration of Commercial Glucose (Corn Syrup) and d-Glucose to Phlorhizinized Dogs.

Experiment No.	Weight of administered substance.		Weight of extra urinary sugar.		Given substance recovered as *d*-glucose.		Remarks.
	Chemically pure *d*-glucose (Kahlbaum).	Water-free commercial glucose.	After *d*-glucose.	After commercial glucose.	*d*-Glucose.	Commercial glucose.	
	gm.	*gm.*	*gm.*	*gm.*	*percent*	*percent*	
1.....	15	16	10.99	12.19	73.27	76.19	Oral administration.
2.....	8	8	4.19	4.01	52.38	50.13	" "
3.....	16	16	12.06	11.87	75.38	74.19	" "
4.....	16	16	13.80	12.57	86.25	78.56	*d*-Glucose subcutaneously; commercial glucose by mouth.
5.....	16	16	11.97	10.69	74.81	66.81	Oral administration.
Total.	71	72	53.01	51.33	74.66	71.29	" "

DISCUSSION.

It will be noted that in all five experiments there were given 71 gm. of pure *d*-glucose, of which 53.01 gm., or 74.7 per cent, reappeared in the urine as extra sugar. The highest percentage of any single dose recovered was 86.25 per cent in Experiment 3, the lowest 52.38 per cent in Experiment 2. In other words, the control doses of *d*-glucose did not reappear quantitatively in the

urine. We have previously obtained results of this sort following doses of substances which, according to the literature, should reappear quantitatively in the urine. A number of possibilities might be suggested in explanation: 1. Some of the glucose might be destroyed in the bowel by bacteria; accordingly, in Experiment 4 the material was given subcutaneously. The yield of extra sugar was higher in this case but still not quantitative. 2. The completeness of the phlorhizination might be questioned, but this possibility we have repeatedly tested and eliminated. 3. Some glucose might be retained in the body and burned. This process might be favored if the rate at which sugar entered the body exceeded that at which it was eliminated, since in such a contingency the concentration of sugar in the tissues (including the blood) would tend to rise and favor oxidation. We have found that too concentrated and rapidly given doses of sugar are as a matter of fact less likely to be quantitatively excreted than slowly given dilute doses, also that it is easier to obtain quantitative returns from starch than from sugar. These observations favor the view that some sugar is retained or burned in the body. We have also observed that more nearly quantitative results were formerly obtained at a time when we had been using Lusk's older method of giving phlorhizin dissolved in carbonate solution at 6 hour intervals, instead of by the Coolen method as at present. In the older method the dogs thus received alkali which was omitted in the later work. We have accordingly tried maintaining a free diuresis through administration of salines supplemented by suitable quantities of alkali during the experiment, and in this way the best yields have been obtained. This seems to suggest that such failures to obtain quantitative returns as those demonstrated in this series may be due to relative impairments of the elimination rate, caused by the effects of acid on the kidneys and other tissues. However this may be, the value of the present series of experiments as throwing light on the utilizability of commercial glucose is maintained by the parallelism between the experiments with this substance and the *d*-glucose controls.

A comparison of the returns from the pure glucose with those obtained from equivalent weights of commercial glucose for all five experiments shows that they stand in the ratio of 74.6 per cent for the pure substance to 71.29 per cent for the commercial

glucose, the dry substance in the latter yielding therefore in all 95.48 per cent as much extra sugar as the same weight of pure *d*-glucose. On the basis that the sample of commercial glucose submitted for study contains 20 per cent of water, 100 gm. of the solid residue is capable of affecting the urinary output of *d*-glucose from completely phlorhizinized and glycogen-free dogs in the same manner as though it contained 95.48 per cent of pure *d*-glucose. This implies that at least 95.48 per cent of the solid matter in the sample of commercial glucose studied is capable of passing through whatever physiological processes of digestion, absorption, and assimilation are necessary for its ultimate existence in the body as *d*-glucose.

The animals showed no evidences of any deleterious effects attributable to the ingestion of commercial glucose, notwithstanding the large size of the doses (1 to 2 gm. per kilo of body weight in 3 to 6 hours), and the great sensitiveness of completely diabetic animals to toxic influences.

The experiments are considered of interest as showing the possibility of using the diabetic organism for resolving a heterogeneous mixture of carbohydrates and allied bodies into the form of a single easily measured sugar, and as casting light on the composition and utilizability of that mixture of glucose, dextrins, and other substances known as commercial glucose.

EXPERIMENTAL PART.

Animals.—Healthy adult female dogs which had borne pups and which weighed 10 to 16 kg. were used exclusively. They were kept in metabolism cages with water but no food.

Collection of Urine.—The urine was drawn by catheter and the bladder flushed out with warm water at 6 hour intervals, the process ending with the withdrawal of the catheter after the flushing and sharply at the end of each period. Additional catheterizations were performed when the animals showed a tendency to urinate in the cage between the regular 6 hour catheterization periods. The urine for each period was collected in a liter volumetric flask and made up to the mark with distilled water.

Drugs.—The phlorhizin used was Merck's. The epinephrin was Parke, Davis and Company's adrenalin in ampules, of which a fresh one was used for each dose.

Phlorhizination.—1 gm. of phlorhizin was triturated with 20 cc. of olive oil and injected subcutaneously at the beginning of the experiment and once every 24 hours thereafter throughout the experiment.

Deglycogenation.—Epinephrin, 0.04 mg. per kilo of body weight, subcutaneously, was given once every 3 hours until G:N was constant. As soon as a constant output of glucose and nitrogen and a constant G:N were attained, the epinephrin was discontinued in accordance with a method described by us in a previous paper.[1]

Analytical Methods.—Glucose in the urine was determined by the titration method of Bang and Bohmannson and by the polariscope, at intervals also both before and after fermentation of the urine with ordinary yeast. Nitrogen was determined according to Kjeldahl.

Commercial Glucose.—The material used was an almost clear, colorless, heavy syrup known as commercial glucose or corn syrup, obtained in the open market. Of this a 10 per cent solution was made which showed in the polariscope a reading of $_{[10]} + 11.95°$. By titration according to Bang's method the original material had a reducing power corresponding to a 36.5 per cent solution of *d*-glucose. It contained 20 per cent of water.

For administration, the material was weighed into 10 or 20 gm. portions, diluted with 10 volumes of water, and given by mouth in several small fractions at ½ to 1 hour intervals. 20 gm. of the original syrup have been reckoned as 16 gm. of water-free substance, on the basis of a 20 per cent H_2O content.

Chemically Pure d-Glucose.—A white, crystalline Kahlbaum's "Traubenzucker" preparation was used. The substance after pulverization and drying was weighed out, made up to 8 per cent strength with water, and the resulting solution checked by titration and the polariscope. The dose of *d*-glucose was then meas-

[1] Sansum, W. D., and Woodyatt, R. T., Studies on the Theory of Diabetes. V. Narcotic Drugs in Phlorhizin Diabetes, *Jour. Biol. Chem.*, 1915, xxi, 1.

ured with a volumetric pipette and administered in the same manner as the corn syrup.

Tabular Record of Experiments.—These show the course of the experiments by 6 and 12 hour periods of time. The term "extra glucose" is always reckoned as $G - (N \times G:N)$, in which G represents the grams of urinary glucose for the period in question; N, the grams of urinary nitrogen; and G:N, the quotient obtained by dividing the grams of glucose for the fore-periods by the grams of nitrogen for the same periods.

EXPERIMENT 1.

Period.	Length of period.	Glucose.	Nitrogen.	G:N.	Extra glucose.	Remarks.
	hrs.	*gm.*	*gm.*		*gm.*	
1....	24	Preparation period.				
2....	6	15.66	3.36	4.66		
3....	6	14.14	4.66	3.03		
4....	6	11.62	4.16	2.79		
5....	6	12.66	4.70	2.70		
6....	6	*19.31*	*3.49*	*5.53*	*12.19*	20 gm. commercial glucose.
7....	6	11.85	3.42	3.46		
8....	6	*16.88*	*2.63*	*6.42*	*10.99*	15 gm. Kahlbaum's glucose.
9....	6	10.00	3.16	3.15		
10....	6	10.05	3.39	2.96		

EXPERIMENT 2.

1....	24	Preparation period.				
2....	6	5.55	1.99	2.79		
3....	6	5.44	1.96	2.87		
4....	6	*8.24*	*1.58*	*5.22*	*4.01*	10 gm. commercial glucose.
5....	6	5.09	1.76	2.89		
6....	6	4.80	1.93	2.49		
7....	6	*8.07*	*1.51*	*5.34*	*4.19*	8 gm. Kahlbaum's glucose.
8....	6	4.91	1.64	2.99		
9....	6	4.91	1.81	2.71		
10....	6	5.26	1.85	2.84		

EXPERIMENT 3.

Period.	Length of period.	Glucose.	Nitrogen.	G:N.	Extra glucose.	Remarks.
	hrs.	*gm.*	*gm.*		*gm.*	
1....	24	Preparation period.				
2....	6	8.23	3.23	2.55		
3....	6	7.40	3.04	2.43		
4....	6	*17.23*	*2.53*	*3.81*		
					11.87	20 gm. commercial glucose.
5....	6	5.85	1.97	2.97		
6....	6	*16.29*	*2.00*	*8.14*		
					12.06	16 gm. Kahlbaum's glucose.
7....	6	5.71	1.99	2.86		
8....	6	5.86	2.31	2.51		

EXPERIMENT 4.

1....	24	Preparation period.				
2....	6	14.25	2.69	5.30		
3....	6	10.28	3.89	2.64		
4....	6	10.43	4.09	2.55		
5....	6	10.43	4.21	2.48		
6....	6	*20.01*	*3.37*	*5.94*		
					12.57	20 gm. commercial glucose.
7....	6	9.60	3.39	2.83		
8....	6	*21.34*	*3.42*	*6.24*		
					13.80	16 gm. Kahlbaum's glucose given subcutaneously.
9....	6	8.31	2.87	2.88		
10....	6	7.28	2.81	2.59		

EXPERIMENT 5.

1....	72	Preparation period.				No epinephrin used.
2....	6	10.06	2.55	3.94		
3....	6	8.58	2.07	4.14		
4....	12	*26.53*	*3.92*	*6.77*	*10.69*	20 gm. commercial glucose.
5....	12	*24.54*	*3.11*	*7.89*	*11.97*	16 gm. Kahlbaum's glucose.
6....	12	18.04	4.14	4.35		
7....	12	15.47	4.40	3.73		

CAN CARBON DIOXIDE IN SEA WATER BE DIRECTLY DETERMINED BY TITRATION?[1]

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(Received for publication, November 15, 1915.)

In two recent papers[2] Moore and his collaborators have arrived at startling conclusions regarding the metabolism of marine organisms. The method pursued by Moore and his coworkers in experiments with a variety of sea animals was extremely simple. The animals were kept in closed vessels of different capacity (2.8 to 45 liters), and the content of carbon dioxide and oxygen of the sea water was determined in aliquot portions at the beginning and end of each experiment. The oxygen was measured by the method of Winkler. The carbon dioxide, on the other hand, was determined by a method of titrating the sea water with $\frac{N}{100}$ acid or alkali, using phenolphthalein as indicator.

It is to be regretted that nowhere in his papers does Moore discuss this method of carbon dioxide determination, for the introduction of which he is responsible, and no information is given beyond the statement that the titrations were made with $\frac{N}{100}$ solutions, employing four drops of the indicator for each 100 cc. of the sea water.

With data thus obtained Moore computes the respiratory quotient, or ratio between the produced carbon dioxide and consumed oxygen, which has gained such importance in all metabolism studies. A review of the results recorded in the papers referred to brings out two significant points: first, the wide fluctuations of the quotients in consecutive experiments; and, second, their high values. We can demonstrate this by the random choice of a series of respiratory quotients obtained in experiments with an unidentified fish during 12 hour periods. The quotients were:

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[1] Published by permission of the Commissioner of Fisheries.

[2] Moore, B., Edie, E. S., Whitley, E., and Dakin, W. J., The Nutrition and Metabolism of Marine Animals in Relationship to (a) Dissolved Organic Matter and (b) Particulate Organic Matter of Sea Water, *Biochem. Jour.*, 1911–12, vi, 255. Moore, Edie, and Whitley, The Nutrition and Metabolism of Marine Organisms; the Rate of Oxidation and Output of Carbon Dioxide in Marine Animals in Relation to the Available Supply of Food in Sea Water, *Report on the Lancashire Sea-Fisheries*, Liverpool, 1913, xxii, 297.

1st day	1.38 – 0.98	6th day	1.56 – 0.96
2nd "	1.17 – 1.13	7th "	2.00 – 1.12
3rd "	0.87 – 1.00	8th "	2.00 – 1.30
4th "	1.28 – 1.09	9th "	2.76 – 1.91
5th "	0.99 – 0.95	10th "	1.90 – 0.95

The respiratory quotient which reflects the nature of the combustion in the organism varies under physiological conditions between 0.70 and 1.00, depending upon the relative amounts of fat, protein, or carbohydrate oxidized. Respiratory quotients which lie outside these limits are not necessarily abnormal and usually indicate synthetic processes accompanying the metabolic activity of the organism. The range of variation, however, is more or less narrow, and is occasioned by determinable factors.

The quotients of the above example have an extraordinary range of variation from 0.95 to 2.76. This is more surprising since the fish was fasting and presumably under uniform conditions throughout the experiment. The respiratory quotient in inanition apparently always tends to a low level corresponding to the combustion of protein and fat upon which the organism subsists; this was also found to hold true in the case of fasting fish.[3]

Each of the facts pointed out above would be sufficient reason for questioning the accuracy of Moore's observations. It is, however, his conception of the metabolism of aquatic animals, which he would isolate from the rest of the animal world, that concerns us. Moore considers that he has demonstrated by his researches that high respiratory quotients (even indeterminable ones) are the expression of inherent peculiarities of their metabolism, resulting from changes in the intramolecular complex. To further substantiate his view, Moore directs attention to Vernon's valuable investigation of the gaseous metabolism of lower aquatic organisms, who also records high respiratory quotients. The allusion to Vernon's results is, however, ill chosen, as he observed such quotients on rare occasions and only when the animals were asphyxiated.

We believe that the erroneous conclusion was drawn from undoubtedly inaccurate facts, and that both must therefore be traced to the method of carbon dioxide determination in the sea water. The method of measuring the carbon dioxide by the changes in acidity of the water, though sound in principle, is encumbered with serious complications in a system as complex as that represented by sea water. And it must always be borne in mind that the CO_2 may exist not only in a dissociated condition as HCO_3, but also as a gas in solution. Only the former would be determined by titration.

The difficulty of getting a sharp and unmistakable end-point when titrating with $\frac{N}{100}$ solutions and phenolphthalein is alone

[3] Morgulis, S., Studies on Fasting Flounders, *Jour. Biol. Chem.*, 1915, xx, 37.

sufficient to condemn the method where great accuracy is essential. In a large series of titrations of sea water we have become convinced that many factors, such as the amount of indicator, length of time since the indicator has been added, excessive agitation of the water—all may cause appreciable differences in the titration. Even under conditions as uniform as it was possible to secure, the titration values were subject to considerable fluctuations. From a large number of determinations we obtained the following average results:

	Sea water. cc.	$\frac{N}{100}$ Acid. cc.
	100	1.57
	200	3.94
	400	6.95
Ratio.....	1:2:4	1:2.5:4.4

These average results, however, do not show the extent of the experimental error. This can be better judged from the fact that the maximum and minimum titration values deviate 10 to 15 per cent from the average.

By the nature of the experiments, where animals are kept in unaerated water, the amount of carbon dioxide which may be allowed to accumulate is small, since the supply of dissolved oxygen can last them only for a limited period. Unless the analysis can be made with great accuracy, as the samples employed for titration must necessarily be a small fraction of the total volume, the error will be greatly multiplied in computation.

Leaving out of consideration these purely technical objections we shall turn our attention to the value of the method *per se*. This we attempted to determine in two ways: first, by checking up the results of the titration against those obtained by a method which we will describe presently; secondly, by determining the per cent of known quantities of carbon dioxide added to water, which we were able to recover by either method.

The method which we utilized for checking the results of titration consisted in precipitating the CO_2 in the water with a standard solution of $Ba(OH)_2$ and measuring the excess by titration. With sea water $Ba(OH)_2$ gives a very bulky precipitate which consists, in addition to the barium carbonate, of barium sulphate and probably also of magnesium and calcium hydroxide. But the amount required to saturate completely a given

volume of sea water is easily found out, and by repeated trials extending over several weeks we became convinced that this amount is constant. With this as a basis we added a reasonable excess of the standard $\frac{N}{5}$ $Ba(OH)_2$ to accurately measured samples of water and titrated aliquot portions of the clear supernatant liquid with $\frac{N}{10}$ or $\frac{N}{50}$ acid. The results obtained by this method are still far from being entirely satisfactory, but the method is susceptible of development and we intend to experiment further with it.

Determinations carried out on samples of the same water by the method of direct titration and by the precipitation method gave invariably much higher results by the latter. To study the titration method more effectively we analyzed waters to which known quantities of carbon dioxide had been added. For lack of better means, we did it by weighing a flask with a measured quantity of water, before and after passing a current of carbon dioxide through it. The carbon dioxide was made from calcium carbonate and chemically pure hydrochloric acid. The gas was passed through sulphuric acid, then through the sample of water. A few minutes were enough to increase appreciably the weight of the flask. The weighings were made on a balance sensitive to the third decimal. After the water was weighed the analyses were made as quickly as possible. From the results of these analyses, given in the subjoined table, the per cent of carbon dioxide recovered was computed.

Water.	CO_2 added.	CO_2 recovered by titration.	
	gm.	*gm.*	*per cent*
Sea water	0.060	0.0257	43.0
	0.130	0.0275	21.2
	0.169	0.0365	21.6
	0.058	0.0172	30.0
Tap water	0.47	0.1530	32.6
	0.73	0.2450	33.6
	0.06	0.0207	34.5
	0.18	0.0564	31.3
	0.26	0.1181	45.4
	0.11	0.0593	54.0
Distilled water	0.022	0.0040	18.0
	0.051	0.0104	20.3

We may conclude from these data that on the average not more than 33 per cent of the carbon dioxide can be recovered by titration. In that case, provided the oxygen determinations were correct, the respiratory quotients ought to have been abnormally low. We cannot reconcile our findings concerning the inefficiency of the method with the high respiratory quotients which Moore obtained by it. In a series of experiments which we made with several species of fish (minnow, cunner, scup, mackerel) in which we measured the oxygen consumption by Winkler's method and the carbon dioxide by titrating with $\frac{N}{100}$ alkali, as Moore did, we found respiratory quotients varying from 0.19 to 1.14, the low quotients being by far more predominant. But no results obtained by a method of such questionable value are of importance, nor can general conclusions derived from them receive serious consideration.

THE STABILITY OF THE GROWTH-PROMOTING SUBSTANCE IN BUTTER FAT.[1]

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A considerable number of observations are now on record to show that certain mixtures of isolated food substances furnishing a ration upon which animals (albino rats) decline or cease to grow can be converted by the addition of some of the natural "fats" into a ration adequate for growth.[2] We have found that the inefficiency of lard and some other fats in this respect is not due to the destruction of the growth-promoting factor by heat, since these fats fail to promote growth adequately even when they are prepared at low temperatures in the laboratory.[3] Furthermore we found that butter fat does not lose its growth-promoting potency by treatment with live steam;[4] and the yolk extracts of heated eggs have also been reported as effective.[5] From such

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[2] McCollum, E. V., and Davis, M., *Jour. Biol. Chem.*, 1913, xv, 167. Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1913, xv, 311; 1913–14, xvi, 423. McCollum and Davis, *Proc. Soc. Exper. Biol. and Med.*, 1914, xi, 101. Osborne and Mendel, *Jour. Biol. Chem.*, 1914, xvii, 401. McCollum and Davis, *Jour. Biol. Chem.*, 1914, xix, 245. Osborne and Mendel, 1915, xx, 379; *Proc. Soc. Exper. Biol. and Med.*, 1915, xii, 92. McCollum and Davis, *Jour. Biol. Chem.*, 1915, xx, 641; xxi, 179. See also Wheeler, R., *Jour. Exper. Zool.*, 1913, xv, 209. MacArthur, C. G., and Luckett, C. L., *Jour. Biol. Chem.*, 1915, xx, 161.

[3] Osborne and Mendel, *Jour. Biol. Chem.*, 1914, xvii, 401; 1915, xx, 380.

[4] Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xx, 381.

[5] McCollum and Davis, *Proc. Soc. Exper. Biol. and Med.*, 1914, xi, 101

facts it seems unlikely that the explanation of the ultimate failure of growth when certain of the natural fats supply the fat component of the diet is to be found in some destructive reaction brought about by preliminary heating. McCollum and Davis[6] believe that the substance which exerts a stimulating action on the growth of rats is sufficiently stable to withstand conditions of saponification which they have employed.

We have found[7] that by fractional crystallization from alcohol it is possible to concentrate the growth-promoting factor present in butter fat and beef fat. It remains in the mother liquor or "oil" fractions, whereas the fractions containing the fats with high melting points are ineffective. In order to learn whether the growth-promoting substance retains its physiological potency when kept for long periods, samples of butter fat and butter "oil," prepared in accordance with published directions,[7] were stored under different conditions. Their efficiency in restoring growth, etc., was subsequently tested on animals that had failed on the "lard diets." It seemed not unlikely that after remaining a long time in the light at room temperatures changes in the physiologically active component of natural fats and oils might ensue such as would render them less potent than before in relation to growth. The feature here involved has a practical bearing in relation to the storage of butter and other fat products.

A quantity of butter "oil" was prepared on September 25, 1914, from part of a lot of butter fat which had previously been found efficient in promoting growth according to our usual experimental procedure. Samples of the butter fat as well as the butter "oil" were preserved:

No. 1 at 18° C., in the light (18° L't).
No. 2 at 18° C., in the dark (18° D'k).
No. 3 at 8° C., in the dark (8° D'k).

The diets of the rats were composed of:

	per cent
Casein	18
Starch	24
"Protein-free milk"	28
Lard	30

[6] McCollum and Davis, *Jour. Biol. Chem.*, 1914, xix, 245.
[7] Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xx, 379.

A part of the lard was replaced by the butter fat or butter "oil" in the experimental recovery period.

The outcome with the *butter "oil"* was as follows: The sample 18° L't still showed potency in November, 1914. A cessation of growth was stopped, continuance of growth was attained, and the attending sore eyes[8] were healed, in Rat 2414 ♀. In March, 1915, the same sample occasioned only transitory improvement and did not stop the early cessation of growth and decline of body weight in Rat 2508 ♂. Butter fat produced a satisfactory recovery. In July, 1915, failure to restore growth was recorded (Rat 2804 ♀). The "sore eyes" were not healed. Here again fresh butter fat produced a good response.

Butter "oil" 8° D'k was still potent in effecting restoration of growth and healing the "sore eyes," in December, 1914 (Rat 2422 ♀). In August, 1915, this potency was no longer manifest (Rat 2921 ♂); but the animal was restored by addition of butter fat 8° D'k to the diet.

With the *butter fat* samples the results were different. All of them, 18° L't, 18° D'k, and 8° D'k, are still efficient, like fresh butter fat, at the present time (December, 1915), more than a year after their preparation.

The foregoing experience, to which little would be added by graphic presentation of the curves of body weight, indicates the pronounced stability of the growth-promoting substance as contained in butter fat under ordinary conditions of storage. However, in the butter "oil," in which the growth-promoting factor is more concentrated than in the original fat, gradual deterioration occurred, so that within a year this characteristic potency was eventually almost completely lost.

[8] Cf. Osborne and Mendel, *Jour. Biol. Chem.*, 1913–14, xvi, 431.

CEPHALIN.

II. BRAIN CEPHALIN.[1]

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In recent years a number of important contributions to the chemistry of cephalin have appeared, particularly those by Parnas and his coworkers.[2] Through the efforts of these workers the principal components have been identified as cephalinic acid ($C_{18}H_{32}O_2$), stearic acid, aminoethyl alcohol, glycerol, and phosphoric acid. Assuming that the molecule of cephalin is composed of equimolecular proportions of these components, we may expect the following composition of the substance:

$C_{41}H_{78}NPO_8$, with C = 66.17; H = 10.57; N = 1.88; P = 4.17

There is, however, a considerable discrepancy between these theoretical values and those found by most observers. The discrepancy, of course, could be explained by the fact that the physical properties of cephalin are of a nature which does not permit the preparation of a sample in sufficient purity. This view, however, is not tenable in the light of our experience with lecithin. Lecithin, both in physical and chemical properties, resembles cephalin closely, and yet the empirical analytical values obtained on the substance by various observers always agree with the theory.

On the other hand, if the empirical composition is correct then the present information regarding the nature of the components of cephalin has to be regarded as incomplete.

In view of this the work on cephalin was resumed, principally from the two following standpoints: (1) Is the composition of ceph-

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913–14, xvi, 419.

[2] Parnas, J., *Biochem. Z.*, 1909, xxii, 411. Baumann, A., *ibid.*, 1913, liv, 30. Renall, M. H., *ibid.*, 1913, lv, 296.

Author.	Source.	C	H	N	P	NH_2	Me
Thudichum[3]	Brain	60.00	9.38	1.68	4.27		
Zuelzer[4]	Ox brain	60.20	9.80	3.80	2.60		
Koch[5]	Sheep brain	59.50	9.80	1.75	3.85		1.73
Cousin[6]	Brain			1.82	3.89		
Stern and Thierfelder[7]	Egg yolk	59.68	9.74	1.57	3.64		
Falk[8]	Nerves	55.75	9.66	1.94	4.42		
	Human brain	57.56	9.21	2.93	3.23		
Neubauer[9]	Brain	61.99	9.85	1.65	3.44		
		62.12	9.87	1.69	3.45		
Parnas[10]	Brain			1.83	3.86		
Frank[11]	Liver	57.10	9.62	1.72	3.91		
Baumann[12]	Human brain			1.84		1.63	
Renall[13]	Ox brain			1.69	3.56	1.37	
	Sheep brain			1.92	4.27	1.60	
Bürger and Beumer[14]	Erythrocytes			1.78	4.06		

alin constant? (2) Do the quantities of the components obtained on hydrolysis agree with those required by the theory, if the prevailing hypothesis of the structure of cephalin is correct?

By varying the nature of the organic solvents and of the temperature at which precipitation or extraction took place we could not change the composition of the resulting phosphatide. Attempts were then made to purify the substance through the lead salt. The analysis of the lead salt gave values for cephalin identical with those of the original substance. When the free cephalin was liberated from the lead salt it again manifested a composition identical with that of the original material. Finally,

[3] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884, 57.

[4] Zuelzer, G., *Z. f. physiol. Chem.*, 1899, xxvii, 262.

[5] Koch, W., *Z. f. physiol. Chem.*, 1902, xxxvi, 136.

[6] Cousin, H., *Compt. rend. Soc. de biol.*, 1906, lxi, 23.

[7] Stern, M., and Thierfelder, H., *Z. f. physiol. Chem.*, 1907, liii, 381.

[8] Falk, F., *Biochem. Z.*, 1908, xiii, 163; 1909, xvi, 190.

[9] Fränkel, S., and Neubauer, E., *Biochem. Z.*, 1909, xxi, 321.

[10] Parnas, *Biochem. Z.*, 1909, xxii, 411.

[11] Frank, A., *Biochem. Z.*, 1913, l, 273.

[12] Baumann, *Biochem. Z.*, 1913, liv, 30.

[13] Renall, M. H., *Biochem. Z.*, 1913, lv, 296.

[14] Bürger, M., and Beumer, H., *Biochem. Z.*, 1913, lvi, 450.

the free phosphatide obtained through the lead salt was reconverted into the lead salt, and this again did not alter the composition of the resulting substance. On the basis of this, one is inclined to conclude that so called cephalin contains some other substance besides the enumerated components. The results of the quantitative hydrolysis of a purified sample showed that the sum of the found components averaged only 90 per cent of the original weight of the substance.

There is nothing to force the conclusion that the substance unaccounted for is an integral part of the molecule. It may be an impurity. We, therefore, concluded to make an attempt to convert cephalin into a saturated phosphatide by reduction with hydrogen after Paal. These experiments were under way before the appearance of Paal's work on the hydrogenation of lecithin.[15] Unfortunately the behavior of the substance towards the reducing agent is very disappointing.

The reduction of cephalin proceeds at a very slow rate, so that for the present we are in possession of only small quantities of material.

The samples thus far analyzed gave analytical values analogous to those of the original material. However, the experience on sphingomyelin has shown that often thirty recrystallizations are required before the substance is obtained in a sufficient degree of purity. Hence we hope to reach a conclusion regarding the true composition of cephalin through further purification of the hydro derivative. Work towards that end is in progress.

EXPERIMENTAL PART.

Preparation of Cephalin.

The cephalin-lecithin fraction of the brain, representing the ether-soluble portion of the alcoholic extract, which still contained some of the cerebroside fraction (cerebrin and sphingomyelin) and some cholesterol, was first purified by precipitating a concentrated ether or petroleum ether solution with dry acetone and then repeatedly with 95 per cent alcohol. After the second or third precipitation from alcohol the precipitate contained little

[15] Paal, C., and Oehme, H., *Ber. d. chem. Ges.*, 1913, xlvi, 1297.

or no ether-insoluble material (cerebroside fraction). The composition of the cephalin did not change after the fifth or sixth precipitation. This material was then either twice suspended in absolute acetone and stirred vigorously for about 2 hours to completely dehydrate it, or better, precipitated two or three times from a concentrated ether solution with dry acetone. Under these conditions the cephalin precipitated as a slightly yellow granular powder, which gradually darkened upon drying, forming a light or dark brown powder. This was easily pulverized. While the moist cephalin is hygroscopic the dried product is stable and easily handled. Prepared in this way cephalin contains a varying amount of ash.

An ash-free preparation may be obtained as follows: The cephalin is emulsified with water by grinding it with a small amount of water in a mortar to a fine paste and then adding water, with stirring, until a thin emulsion results. This is then precipitated by adding 10 per cent hydrochloric acid until no further precipitate forms. The cephalin separates as a light yellow amorphous mass which filters slowly with suction. It is best separated from the water by centrifuging the mixture. The moist product is taken up in dry ether and the solution either dried with sodium sulphate, concentrated, and precipitated, or better, concentrated at once and precipitated with dry acetone.

The same object is more rapidly accomplished by decanting off most of the dilute acid, filtering the cephalin over cheesecloth, and extracting the cephalin with ether. The difficulty of extracting with ether, in that it forms a gelatinous emulsion,[16] is overcome by the addition of absolute alcohol, care being taken that the amount added does not precipitate the cephalin.

In either case repeated precipitation is necessary to obtain an anhydrous product. This method is accompanied by considerable loss of material, because of the solubility of the cephalin in the acidulated water.

The same object is obtained, probably with less loss of material, by shaking an ether solution of cephalin with 10 per cent hydrochloric acid solution several times, according to Parnas,[17] then

[16] Thudichum, *loc. cit.*, 58.

[17] Parnas, *loc. cit.*

with water, and precipitating as above. The emulsions which may result are best prevented by the addition of absolute alcohol. Such a sample gave the following figures upon analysis:

0.1542 gm. substance gave 0.3420 gm. CO_2 and 0.1312 gm. H_2O.
0.1516 " " " 0.3342 " " " 0.1250 " "
0.5000 " " neutralized 7.0 cc. $\frac{N}{10}$ HCl.
0.2000 " " in acetic acid, gave 6.4 cc. N gas at 20° and 750 mm.
0.3000 " " gave 0.0410 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{78}NPO_8$:	Calculated[18] for $C_{41}H_{78}NPO_{13}$:	Found:
C	66.17	60.00	60.49
			60.13
H	10.57	9.55	9.52
			9.22
N	1.88	1.70	1.96
NH_2 N	1.88	1.70	1.79
P	4.17	3.76	3.80

The formula $C_{41}H_{78}NPO_8$ is based on the assumption that the molecule contains 1 molecule each of aminoethyl alcohol, $H_2NCH_2CH_2OH$, glycerophosphoric acid, $C_3H_5(OH)_2OPO(OH)_2$, stearic acid, $C_{18}H_{36}O_2$, and cephalinic acid, $C_{18}H_{32}O_2$. As is seen from the analytical figures, and as has already been mentioned in the introduction, there is a marked discrepancy between these figures and the calculated ones. The formula $C_{41}H_{78}NPO_{13}$ agrees much better with the analytical figures found.

Attempts to Purify Cephalin.

Thinking that perhaps our methods of purification were not sufficiently rigorous, we submitted our material to the following treatments.

A portion of cephalin was dissolved in boiling amyl alcohol (50 gm. require about 250 cc. for solution) and the solution quickly cooled. The cephalin separated as a thick, amorphous mass. This was ground up with dry acetone and repeatedly washed with the same solvent.

A second lot was dissolved in hot ethyl acetate (10 gm. require about 200 cc. for solution). Upon cooling the solution in the ice box the cephalin precipitated out nearly quantitatively.

[18] Fränkel and Neubauer, *Biochem. Z.*, 1909, xxi, 321.

This was washed with dry acetone as above. The composition of the cephalin in either case remained unchanged, so that there was no purification by these methods. This is in agreement with the observations of Baskoff[19] on liver heparphosphatide.

We then examined the method used successfully by MacLean[20] in obtaining cuorin. This consists in extracting the crude material with alcohol at 60–70° for several hours and repeating the process several times. Finding that this operation did not give us any purification, we modified the method as follows. The cephalin was dissolved in a small amount of gasoline, boiling at 50–60°, or in ether, and poured into 98 per cent alcohol which was kept at about 60° during the operation. A part of the cephalin settles out as a thick oil on the bottom and sides of the flask and on the stirrer, while a part remains in the alcohol-gasoline or the alcohol-ether mixture. The precipitate is taken up in ether and precipitated with acetone. The solution is concentrated in vacuum, the residue taken up in ether and precipitated. Since the two lots of material have the same composition no fractionation was effected. The following figures were obtained upon analysis of the fraction which remained in the alcohol-ether mixture:

0.1482 gm. substance gave 0.3260 gm. CO_2 and 0.1274 gm. H_2O.
0.5000 " " neutralized 6.33 cc. $\frac{N}{10}$ HCl.
0.3000 " " gave 0.0388 gm. $Mg_2P_2O_7$.

	C	H	N	P
Found:	60.00	9.62	1.78	3.60

Properties of Cephalin.

There is little to add to the many properties already given by Thudichum, Falk, Parnas, and Fränkel. It may be well to call attention to certain differences which have been noted in the literature. Thudichum states that cephalin is soluble in anhydrous ether, while Parnas[17] says it is not. We have found that perfectly anhydrous ether does not dissolve cephalin, but remains clear for some time. A drop of water added to the ether at once produces the characteristic deep red, fluorescent solution.

[19] Baskoff, A., *Z. f. physiol. Chem.*, 1908, lvii, 395.

[20] MacLean, H., *Z. f. physiol. Chem.*, 1908, lvii, 304; *Biochem. Jour.*, 1909, iv, 168; 1912, vi, 333.

The above mentioned method of purification shows that cephalin is markedly soluble in warm 98 per cent alcohol (it does not precipitate out when the ether is removed). Koch and Falk state that it is insoluble in cold or hot alcohol. This may be influenced by the fact that the cephalin is added as an ether solution; the same concentration cannot be reached by boiling cephalin with alcohol. That cephalin is somewhat soluble in cold alcohol is easily seen in the loss on repeated precipitation.

The precipitation of cephalin from an aqueous emulsion or suspension by hydrochloric acid is probably a process of coagulation and does not depend upon the formation of a hydrochloride.[21] Fränkel and Neubauer[18] express the same view and show that the same precipitation is brought about by organic acids and mineral salts.

Cephalin may be obtained in suspension as a nearly colorless substance by precipitation with hydrochloric acid. All attempts to dry it in such a way as to retain this color have failed. As soon as the water is decanted off and acetone is added, the brown color appears. If ether is used, this brown color appears instantly. The color seems to have no influence upon the composition of the molecule.

Lead Compound of Cephalin.

Since the various methods of fractional precipitation and extraction of cephalin failed to give us a product that corresponded to the one theoretically expected, we attempted a purification by chemical means. The most promising method seemed to be through the lead compound. Such a derivative of cephalin (peroxycephalin) has been described by Thudichum.[22] He prepared it by treating an ether solution of cephalin with a warm alcoholic solution of lead acetate and purified it by precipitating from ether with alcohol.

The lead compound of cephalin was prepared as follows: 20 gm. of cephalin were dissolved in 200 cc. of boiling amyl alcohol and this hot solution was treated with a boiling solution of about 25 gm. of lead acetate in amyl alcohol. The deep red solution was then allowed to cool nearly to room temperature and an ex-

[21] Thudichum, *loc. cit.*, 53.
[22] Thudichum, *loc. cit.*, 139.

cess of methyl alcohol added, with stirring, which precipitated the lead compound as a light yellow amorphous powder. This can be easily dried and is not hygroscopic. The first samples prepared seemed to have a nitrogen to phosphorus ratio of 1:2, but later preparations, carefully purified, had approximately the same ratio as the original cephalin. The material is purified by thoroughly extracting with boiling methyl alcohol, 80 per cent acetone, and dry acetone. Before purification a part of the lead salt is soluble in ether. The soluble and insoluble parts appeared to have the same composition. After precipitation of the ether-soluble fraction by adding about two volumes of methyl alcohol and extraction of the precipitate with dry acetone, the product is no longer ether-soluble. Thus the apparent solubility in ether was probably due to some impurity. The lead compound is insoluble in cold or warm alcohol, acetone, or ether, more or less soluble in benzene, toluene, and pyridine. Upon cooling the hot amyl alcohol solution deposits the unchanged lead salt as an amorphous mass. The amyl alcohol may be removed by washing with dry acetone. Glacial acetic acid does not completely dissolve the lead salt; it seems likely that a part of the lead is split off during the process of solution, since the residue from the extraction with acetic acid was found to contain lead acetate. A few of the many analyses are given below:

A. 0.1482 gm. substance gave 0.2154 gm. CO_2, 0.0790 gm. H_2O, and 0.0622 gm. ash.
0.5000 gm. substance neutralized 3.16 cc. $\frac{N}{10}$ HCl.
0.3000 " " gave 0.0252 gm. $Mg_2P_2O_7$.
B. 0.1498 " " " 0.2188 gm. CO_2, 0.0840 gm. H_2O, and 0.0634 gm. ash.
0.5000 gm. " neutralized 3.11 cc. $\frac{N}{10}$ HCl.
0.3000 " " gave 0.0294 gm. $Mg_2P_2O_7$.
C. 0.5000 " " neutralized 3.21 cc. $\frac{N}{10}$ HCl.
0.3000 " " gave 0.0262 gm. $Mg_2P_2O_7$.
D. 0.5000 " " neutralized 3.01 cc. $\frac{N}{10}$ HCl.
0.3000 " " gave 0.0276 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{78}NPO_8Pb_2$:	Calculated for $C_{41}H_{74}NPO_{13}Pb_2$:	Found: A.	B.	C.	D.	Thudichum.
C.....	42.65	39.82	39.64	39.84			38.37
H.....	6.46	6.03	5.81	6.27			5.76
N.....	1.21	1.13	0.88	0.87	0.90	0.84	0.97
P.....	2.68	2.50	2.34	2.73	2.43	2.59	2.72
Ash...			41.97	42.40			

Preparation of Cephalin from the Lead Compound.

In order to determine whether the preparation and purification of the lead compound had brought about any purification of the cephalin itself, the lead compound was reconverted into the free cephalin. 50 gm. of the material were dissolved in a mixture of 200 cc. of toluene, 100 cc. of benzene, and 50 cc. of pyridine by warming a short time on the water bath. The lead was removed by means of hydrogen sulphide, the lead sulphide filtered off, and the clear solution concentrated to dryness in vacuum. The residue was taken up in ether and precipitated repeatedly with dry acetone. As the analyses show, this material had practically the same composition as the original cephalin used for making the lead salt; this indicates that the lead process is not one of purification.

0.1488 gm. substance gave 0.3276 gm. CO_2 and 0.1278 gm. H_2O.
0.5000 " " neutralized 6.84 cc. $\frac{N}{10}$ HCl.
0.3000 " " gave 0.0388 gm. $Mg_2P_2O_7$.

	C	H	N	P
Found:	60.05	9.61	1.87	3.60

This process is, at best, very unsatisfactory. The preparation of the lead salt is not quantitative, and the removal of the lead is accompanied by great loss of material. The operations must be carried out as rapidly as possible, for if cephalin or its lead compound is allowed to stand for some time in a benzene-pyridine solution, even at 0°, a certain decomposition occurs, in which the nitrogen content is increased. The nature of this change is being further investigated.

Two samples of cephalin, purified through the lead salt, were again converted into the lead compound as given above. The same lead compound was obtained, again showing that the cephalin thus obtained was identical with that used for the preparation of the original lead salts.

0.5000 gm. substance neutralized 3.4 cc. $\frac{N}{10}$ HCl.
0.5000 " " " 3.46 " " "
0.3000 " " gave 0.0242 gm. $Mg_2P_2O_7$.
0.3000 " " " 0.0260 " "

	N		P	
Found:	0.95	0.97	2.24	2.41

Quantitative Hydrolysis.

As the base and the glycerophosphoric acid of cephalin are water-soluble, it is comparatively easy to determine the amount of fatty acid obtained upon hydrolysis. 5 gm. of cephalin were boiled with 300 cc. of 3 per cent sulphuric acid for 24 hours. Upon cooling 0°, the fatty acids solidified. These were filtered off, washed free of acid by melting over water several times, and then taken up in dry acetone. By evaporating off the acetone several times the product was dehydrated. It was finally dried to constant weight at 100°.

4.75 gm.	cephalin	gave	2.970 gm.	fatty	acid.
2.75 "	"	"	3.026 "	"	"
5.0 "	"	"	3.202 "	"	"

	Fatty acid. *per cent*		
Found:	62.6	63.7	64.0

Glycerol Estimations.

Koch believed that cephalin contained one methyl group attached to nitrogen, because when heated with hydriodic acid one molecule of methyl iodide was formed under 240°. Similar results were obtained by Fränkel and Neubauer. Renall also reports the formation of methyl iodide when cephalin was heated to 360°, but states that the amount obtained was not equivalent to the ratio N:Me = 1:1. Recent work makes it appear evident that this formation of methyl iodide was really due to the presence of glycerol.

Foster[23] has shown that glycerol may be readily determined in lipoids by the use of the original Fanto-Zeisel method. By using a modified Herzig-Meyer apparatus it is possible to determine both the glycerol and the methyl attached to nitrogen.

We have used the Fanto-Zeisel method for the determination of glycerol in cephalin with satisfactory results. The reaction proceeds smoothly, with little or no foaming, and is complete in about 2 hours if the temperature of the metal bath is maintained at 125–128°. Blank determinations on a known solution of glycerol in water gave good checks. Experiments with aminoethyl

[23] Foster, M. L., *Jour. Biol. Chem.*, 1915, xx, 403.

alcohol showed that under the conditions of the reaction no decomposition (with the formation of volatile iodides) took place. 0.3000 gm. of cephalin gave, in three experiments, 0.0748, 0.0760, and 0.0784 gm. of silver iodide respectively. This corresponds to 9.77, 9.93, and 10.2 per cent glycerol. Koch's result, as recalculated by Foster, was 10.8 per cent, while Winterstein and Hiestand found 10.2 per cent. Foster obtained a slightly lower figure, 8.21 per cent. The corresponding figure for lecithin varies from 8.75 to 11.6 per cent glycerol.

Composition of the Cephalin Molecule.

The amount of base (aminoethyl alcohol) in cephalin cannot be estimated directly, because all its derivatives (gold chloride salt, platinic chloride salt, picrate, picrolonate) are very soluble in water or alcohol. However, since all the nitrogen has been shown to be amino nitrogen,[24] we are justified in using the percentage of nitrogen as a basis for the calculation of the amount of base. With 1.9 as the average of the nitrogen content, the amount of aminoethyl alcohol is calculated as 8.26 per cent.

In the same way, with 3.85 as the average phosphorus content, the amount of phosphoric acid is calculated as 12.17 per cent.

In calculating the composition of the molecule the components should add up to 109.7 per cent, since this is the relation between the molecular weight of the formula $C_{41}H_{78}NPO_8$ and the hydrated formula $C_{41}H_{86}NPO_{12}$. The calculated and found values are given below:

	Calculated for $C_{41}H_{78}NPO_8$:	Found:
Base	8.2	8.26
Glycerol	12.4	10.00
Phosphoric acid	13.2	12.17
Fatty acid	75.9	63.40
Totals	109.7	93.83

Thus it is seen, as mentioned in the introduction, that the molecule is not entirely accounted for. As yet we can give no reason for the discrepancy.

[24] Baumann, *Biochem. Z.*, 1913, liv, 30. Renall, *ibid.*, 1913, lv, 296.

Hydrocephalin.

1 gm. of cephalin was dissolved in a mixture of 40 cc. of ordinary ether and 5 cc. of glacial acetic acid and shaken with colloidal palladium in an atmosphere of hydrogen, according to Paal. The hydrogen was absorbed at the rate of about 1 cc. per minute, gradually decreasing as the reaction proceeded. At the end of the reaction 70 cc. (measured at room temperature) had been absorbed, while 1 gm. of cephalin, with the formula $C_{41}H_{78}NPO_{13}$ (mol. wt. 743) should absorb 60 cc. measured under standard conditions. Reduction experiments in pyridine and acetic acid or in amyl alcohol and acetic acid were unsuccessful. The reaction product was concentrated on the water bath until all the ether had been removed, then treated with a large volume of acetone and filtered. The precipitate was taken up in boiling absolute alcohol containing a trace of acetic acid, heated until the palladium was coagulated, and filtered. Upon cooling the reduced cephalin separated out as an amorphous powder. This was purified by washing with ether, dissolving in boiling absolute alcohol, and allowing the product to settle out at 0°. It forms a nearly colorless, non-hygroscopic powder. Because of the small amount at our command we were unable to obtain it crystalline.

Two samples were analyzed. The first was analyzed after the second and third crystallizations, the second after the second crystallization.

A. 0.0998 gm. substance gave 0.2273 gm. CO_2 and 0.0930 gm. H_2O.
0.1062 " " " 0.2420 " " " 0.0958 " "
0.0300 " " " 0.0700 " " " AgI (glycerol estimation).
B. 0.1060 " " " 0.2412 " " " 0.1004 gm. H_2O.

		C	H	Glycerol.
Found:	A.	62.12	10.43	9.15
		62.15	10.10	
	B.	62.06	10.60	

The two samples given above were prepared last year. This fall we again took up the question of obtaining hydrocephalin, but with no better success. The reaction appears to be uncertain; at times the reduction proceeds smoothly, at other times

scarcely any hydrogen is absorbed. We have tried to further purify the cephalin but this does not increase the rate of reduction.

The product obtained this year gave the following figures on analysis:

0.1022 gm. substance gave 0.2256 gm. CO_2 and 0.0912 gm. H_2O.
0.300 " " " 0.0370 " $Mg_2P_2O_7$.
0.200 " " neutralized 3.01 cc. $\frac{N}{10}$ HCl.

	Found:
C	62.25
H	10.34
N	2.22
P	3.62

GLUCOSAMINOHEPTONIC ACID.

By P. A. LEVENE.

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The work of Levene and La Forge[1] has demonstrated that the substitution of the amino group by a hydroxyl in the series of amino sugars may be associated with a change of configuration of the α-carbon atom. This property raises great difficulty in reaching, by simple methods, a conclusion regarding the complete configuration of any amino sugar. The configuration of glucosamine, the most accessible and the best known substance of this group, still remains unknown. Because of the difficulty of arriving at the goal by direct methods, the problem is being approached in this laboratory by several indirect ways, all of which will be discussed later. One of these, however, will be outlined here. In the light of the experience of Fischer[2] and his coworkers it seems possible that a substitution in the β position from the carbonyl group may take place without a Walden rearrangement. If this were to hold true for the 2-aminoheptonic acids, then the solution of the question of the configuration of these substances should be easily accessible. On the other hand, the knowledge of the configuration of the carbon atom carrying the amino group in the heptonic acids will contain in itself the information regarding the configuration of the same group in the parent hexosamines. Hence the first problem demanding solution is the question of the occurrence or non-occurrence of the Walden rearrangement during deamination of the 2-aminoheptonic acids. The derivative of glucosamine was the subject of the present investigation. In order to make this investigation

[1] Levene, P. A., and La Forge, F. B., *Jour. Biol. Chem.*, 1915, xx, 433; xxi, 345, 351; xxii, 331.

[2] Fischer, E., and Scheibler, H., *Ber. d. chem. Ges.*, 1909, xlii, 1219. Fischer, E., Scheibler, H., and Groh, R., *ibid.*, 1910, xliii, 2020.

possible it is necessary to be in possession of a crystalline 2-aminoheptonic acid derived from glucosamine.

An attempt to prepare this heptonic acid was made by Neuberg,[3] who claimed to have obtained an amorphous copper salt, which gave analytical values agreeing with those required by the heptonic acid. Such material could be of little value in our work, especially since the amorphous salts prepared by us gave values only approximating those required by the theory. Hence we renewed the efforts towards obtaining a crystalline glucosaminoheptonic acid. The present communication contains a report of the mode of preparation and of the properties of the acid which has the structure of one of the following two possible epimers.

$$CH_2(OH)\frac{H\quad H\quad OH\quad H\quad H}{OH\quad OH\quad H\quad NH_2\quad OH}COOH \quad \text{or}$$
(?)

$$CH_2(OH)\frac{H\quad H\quad OH\quad H\quad OH}{OH\quad OH\quad H\quad NH_2\quad H}COOH$$
(?)

EXPERIMENTAL PART.

Portions of 25.0 gm. of glucosamine hydrochloride were taken up in 25 cc. of water, 10 cc. of an 80 per cent aqueous solution of hydrocyanic acid added, and this was followed by the addition of 12.5 cc. of ammonia water. The chloride generally dissolved soon after the addition of ammonia. The solution turned dark brown within 2 hours, and on further standing became quite viscous. It was allowed to stand at room temperature for 3 to 4 days. The solution was then taken up in about 400 cc. of water, boiled with a slight excess of lead carbonate, filtered, and precipitated with basic lead acetate and ammonia. The precipitate was decomposed with hydrogen sulphide, filtered, and concentrated to about 300 cc. To the solution about 40.0 gm. of barium hydroxide were added and the mixture was heated over a flame until the greatest part of ammonia was removed. On standing, a brown flocculent precipitate formed and was removed by filtration. The filtrate was then concentrated under

[3] Neuberg, C., *Ber. d. chem. Ges.*, 1902, xxxv, 4009. Neuberg, C., and Wolff, H., *ibid.*, 1903, xxxvi, 618.

diminished pressure to dryness to remove all the ammonia. The residue was taken up in water, and the solution acidulated with sulphuric acid to remove all barium. Omitting filtration, excess of lead carbonate was added and the mixture boiled until, upon a further addition of the reagent, evolution of carbon dioxide was no longer manifested. The reaction product was then allowed to stand at 0°C. over night and filtered. The lead was then removed by means of hydrogen sulphide, the remaining hydrochloric acid by means of silver carbonate, and the excess of silver again removed by hydrogen sulphide. The filtrate was boiled with animal charcoal until it became perfectly colorless. The colorless solution was concentrated to about 75.0 cc. and to this methyl alcohol was added in small portions. The addition of alcohol caused the appearance of a white flocculent precipitate at the place of contact. By manipulation this was slowly brought again in solution, and the addition of alcohol continued until the solution turned slightly opalescent. It was then allowed to stand at least 24 hours at 0°C. A crystalline precipitate appeared, consisting of rosettes composed of needles. The yield was about 30 per cent of the glucosamine hydrochloride used. On recrystallization the substance crystallized in individual needles. The acid has a melting point of 169°C. (corrected). For analysis it was dried in a vacuum desiccator over sulphuric acid at 100°.

0.1004 gm. of substance gave 0.1366 gm. CO_2 and 0.0618 gm. H_2O.
0.0970 " " " required for neutralization 4.38 cc. $\frac{N}{10}$ acid (Kjeldahl).

	Calculated for $C_7H_{15}NO_7$:	Found:
C	37.33	37.10
H	6.66	6.88
N	6.22	6.25

In 2.5 per cent solution of hydrochloric acid the substance had the following rotation:

Initial.

$$[\alpha]_D^{25} = \frac{-0.02° \times 2.168}{0.1 \times 0.150} = -2.89°$$

Equilibrium. (After 24 hours.)

$$[\alpha]_D^{25} = \frac{-0.52° \times 2.168}{0.1 \times 0.150} = -7.52°$$

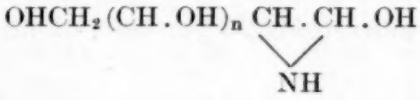
AMMONIA DERIVATIVES OF THE SUGARS.

By P. A. LEVENE.

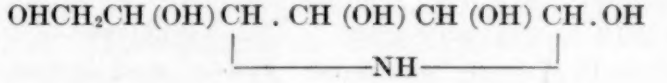
(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 30, 1915.)

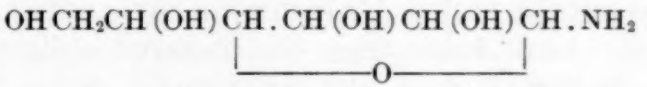
The structure of the so called glucosimines has been the subject of considerable discussion. Lobry de Bruyn,[1] who first discovered them, assigned to them the following structure:



Wohl[2] modified this first conception, accepting the ring between the end and the γ-carbon atoms:



Finally, Irvine, Thomson, and Garret[3] have assumed the glucosidic structure:



The evidence advanced by Irvine and his coworkers is of indirect nature, and more direct evidence supporting their hypothesis seemed desirable. Besides, the knowledge of the structure of these substances has assumed new importance because

[1] Lobry de Bruyn, C. A., and Franchimont, A. P. N., *Rec. d. trav. chim. d. Pays-bas*, 1893, xii, 286. Lobry de Bruyn, *Ber. d. chem. Ges.*, 1895, xxviii, 3082.

[2] Wohl, quoted by von Lippmann, E. O., Die Chemie der Zuckerarten, Braunschweig, 1904, i, 504.

[3] Irvine, J. C., Thomson, R. F., and Garret, C. S., *Jour. Chem. Soc.*, 1913, ciii, 238.

of its bearing on the mechanism of the reaction between hydrocyanic acid and the sugar imines.

In order to prove the glucosidic structure it is necessary to furnish conclusive evidence as to one of two requisites, or to both: first, as to the existence of the γ-oxidic ring in sugars (this structure carries the property of mutarotation); second, as to the presence of an unsubstituted NH_2 group. These two points have been taken into consideration by previous workers, but they received no conclusive answer.

The presence of a primary NH_2 group was tested in this work by comparing the nitrogen values obtained by the Kjeldahl process and by the method of Van Slyke with nitrous acid. It was found that in 4 minutes all the nitrogen of the imine was transformed into nitrogen gas. In the same interval ammonia does not give off more than 20 per cent of its nitrogen as nitrogen gas.[4]

Furthermore, on material which was only slightly hydrolyzed, the values obtained by the Kjeldahl process were higher than those obtained in 4 minutes by the nitrous acid method. In connection with this it may be mentioned that the rate of hydrolysis varies with individual sugars. The derivatives of four sugars were analyzed: glucose, galactose, xylose, and lyxose. Lyxosimine was found the most, and glucosimine the least stable.

Regarding the change in rotation the following was noted. Freshly prepared samples of xylosimine and of lyxosimine, kept in aqueous solution at 0° for 24 hours, did not manifest any change in rotation. In 63 hours there was observed a slight change in rotation and with it there was also noted a change in nitrogen distribution, the nitrogen gas evolved by nitrous acid showing a lower value than the total nitrogen.

On the other hand, if the sample of the dry imine was kept for 7 days over soda lime under diminished pressure and then analyzed, it was found to manifest some mutarotation in 24 hours, even when the nitrogen distribution remained constant. In this sample there was always present a slight excess of the total nitrogen over the amino nitrogen. Hence the mutarotation was undoubtedly due to the presence of little of the free sugar.

[4] Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 281.

Thus, the presence of the primary amino group in the molecule of the sugar imines is demonstrated; on the basis of this one is justified in assigning to the sugar imines a glucosidic structure. The name amino-glucosides would perhaps be more correct.

Table of Results.

	Total N calculated for $C_5H_{11}NO_4$ = 9.40 per cent. Found:		NH_2 N calculated for $C_5H_9O_4.NH_2$ = 9.40 per cent. Found:		
	Immediately.	After 63 hrs.	Immediately.	After 24 hrs.	After 63 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Xylosimine.					
I.....	9.73	9.64	9.39		9.07
II.....	9.06		8.78	8.78	
Lyxosimine.					
I.....	9.64	9.58	9.16		8.38
II.....	8.92		7.83	7.80	

EXPERIMENTAL PART.

I. Xylosimine[5] was prepared according to the method of Lobry de Bruyn and Van Leent.[6] The substance was dried for 24 hours over soda lime under diminished pressure.

0.1000 gm. substance required for neutralization 6.95 cc. $\frac{N}{10}$ acid (Kjeldahl).

0.0500 gm. substance gave in 4 minutes 8.2 cc. N at 20°, 764 mm. (Van Slyke).

The same solution was analyzed after 63 hours' standing at 0°C.

0.1000 gm. substance required for neutralization 6.89 cc. $\frac{N}{10}$ acid.

0.0500 " " gave in 4 minutes 7.9 cc. N gas at 20°, 765 mm.

The rotation of the substance in cold water of about 5°C. (dissolved in water at 0°) was the following:

Immediately.

$$[\alpha]_D^5 = \frac{-0.38° \times 4.1850}{0.5 \times 0.2000} = -15.9°$$

After 63 hours.

$$[\alpha]_D^5 = \frac{-0.38° \times 4.1850}{0.5 \times 0.2000} = -15.9°$$

[5] We take this occasion to express our indebtedness to the Lookout Refining Company, Chattanooga, Tenn., who furnished us free of charge the cottonseed hulls which served for the preparation of the xylose used in this work as well as in the work on "Xylohexosaminic Acid."

[6] Lobry de Bruyn, C. A., and Van Leent, F. H., *Rec. d. trav. chim. d. Pays-bas*, 1895, xiv, 134.

II. Xylosimine was prepared in the same manner and kept for 7 days in a desiccator:

0.1000 gm. substance required for neutralization 6.47 cc. $\frac{N}{10}$ acid.
0.0500 " " gave in 4 minutes 7.7 cc. N gas at 20°, 762 mm.
After 24 hours the same solution gave:
0.0500 gm. substance gave in 4 minutes 7.6 cc. N gas at 19°, 768 mm.
Rotation under the same conditions as above:

Immediately. After 24 hours.

$$[\alpha]_D^5 = \frac{-0.42° \times 4.1630}{0.5 \times 0.2000} = -17.48° \qquad [\alpha]_D^5 = \frac{-0.38° \times 4.1630}{0.5 \times 0.2000} = -15.71°$$

III. Lyxosimine was prepared in the manner described by Levene and La Forge.[7] It was kept in the desiccator over night.

0.1000 gm. substance required for neutralization 6.89 cc. $\frac{N}{10}$ acid.
0.0500 " " gave in 4 minutes 8.0 cc. N gas at 20°, 764 mm.
The same solution after 63 hours gave:
0.1000 gm. substance required for neutralization 6.84 cc. $\frac{N}{10}$ acid.
0.0500 " " gave in 4 minutes 7.3 cc. N gas at 20°, 765 mm.
The rotation was:

Immediately. After 63 hours.

$$[\alpha]_D^5 = \frac{-1.30° \times 4.1950}{0.5 \times 0.2000} = -54.53° \qquad [\alpha]_D^5 = \frac{-1.06° \times 4.1950}{0.5 \times 0.2000} = -44.46°$$

IV. Lyxosimine was prepared as the sample above, but kept in the desiccator 7 days.

0.1000 gm. substance required for neutralization 6.37 cc. $\frac{N}{10}$ acid.
0.0500 " " gave in 4 minutes 6.75 cc. N gas at 19°, 768 mm.
The rotation was:

Immediately. After 24 hours.

$$[\alpha]_D^5 = \frac{-0.72° \times 4.1952}{0.5 \times 0.2000} = -30.25° \qquad [\alpha]_D^5 = \frac{-0.55° \times 4.1952}{0.5 \times 0.2000} = -23.10°$$

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[7] Levene, P. A., and La Forge, F. B., *Jour. Biol. Chem.*, 1915, xxii, 331.

SPHINGOSINE.

IV. SOME DERIVATIVES OF SPHINGOSINE AND DIHYDROSPHINGOSINE.

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(Received for publication, November 19, 1915.)

Sphingosine has generally been identified by the analysis of the sulphate. Some workers have based their conclusions on the presence of sphingosine when the sample possessed the nitrogen content of sphingosine sulphate or, besides the nitrogen content, showed the required melting point. In the course of the work carried out in this laboratory during the last few years the conviction was gained that the composition of the sulphate is not sufficiently constant to justify conclusions on the basis of analytical data for one or two elements. Thus the differences in the nitrogen and sulphur content of the homologues of sphingosine are within the limits of analytical error. Also the melting point does not offer a sufficient guarantee of its purity. Because of this, attempts were made to prepare other salts of sphingosine and dihydrosphingosine. Picric and picrolonic acids were found to give with the two bases salts of constant composition. However, the solubility of these salts in organic solvents is too great to make them very useful for analytical purposes.

This communication contains also a report of further work on the structure of sphingosine. Levene and West[1] have shown that the probable structure of sphingosine is

$$C_{12}H_{25}CH = CH \,.\, CHOH \,.\, CHOH \,.\, CH_2NH_2$$

The isolation of normal pentadecylic acid from the oxidation products of dihydrosphingosine established the nature of the first 15 carbon atoms. There thus remains as unknown the character

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913–14, xvi, 549; 1914, xviii, 481.

of the two end carbon atoms and the relative positions of the hydroxyl and amino groups. Levene and Jacobs[2] first attempted to answer these questions by trying to reduce dihydrosphingosine to the corresponding amine. Using hydriodic acid in sealed tubes at 125°, they obtained an unsaturated compound, sphingamine, $C_{17}H_{35}N$. The same substance was also obtained when the dichloro derivative was reduced with sodium and alcohol. Because of this it was attempted to modify the conditions of reduction of the base by means of hydriodic acid. An interesting observation was made when the process was carried out in glacial acetic acid solution; under these conditions only one hydroxyl group was reduced, leaving a monohydroxymonoamino alcohol. This substance undoubtedly could serve as a starting point for further work on the structure of sphingosine. However, a chance discovery made the material more accessible in another way.

In the course of the present work also the trihydroxy alcohol—dihydrosphingosol—was obtained.

EXPERIMENTAL PART.

Sphingosine Picrolonate.

The sphingosine from 4.5 gm. of sphingosine sulphate, dissolved in a little alcohol, was added to a solution of 3 gm. of picrolonic acid in alcohol. The slightly yellow solution immediately turned a deep red. Boiling water was then added until the solution was turbid; upon cooling the picrolonate separated as a deep red oil, which gradually changed to yellow crystals. It was purified by precipitating several times from an alcoholic solution with boiling water and finally by extracting with ether at room temperature. Sphingosine picrolonate is easily soluble in ethyl alcohol, somewhat soluble in methyl alcohol and acetone, and very slightly soluble in ether. It softens at 81° and melts at 87–89°.

0.1110 gm. substance gave 0.2397 gm. CO_2 and 0.0792 gm. H_2O.

	Calculated for $C_{27}H_{43}N_5O_7$:	Found:
C	58.97	58.90
H	7.88	7.98

[2] Levene, P. A., and Jacobs, W. A., *Jour. Biol. Chem.*, 1912, xi, 547.

Abderhalden and Weil[3] report that they were able to estimate picrolonates by the ordinary Kjeldahl method, only two of the nitrogens of picrolonic acid being converted into ammonia. We tried this method on the above picrolonate but found that the value for N was always high. The following results were obtained:

0.2434 gm. substance required 17.8 cc. $\frac{N}{10}$ HCl.
0.2816 " " " 20.4 " " "
0.2021 " " " 14.7 " " "

	Calculated for 3N:		Found:	
N	7.65	10.24	10.15	10.19

The determination of nitrogen in *dl*-leucine picrolonate[4] was then tried, for which Abderhalden and Weil give: calculated, 10.65; found, 10.90.

0.1924 gm. substance required 17.6 cc. $\frac{N}{10}$ HCl.
0.1777 " " " 15.7 " " "

	Calculated for 3N:	Found:	
N	10.65	12.81	12.38

Blank experiments on picrolonic acid gave values between those calculated for 2N and 3N.

Dibromosphingosine Sulphate.

Sphingosine sulphate, dissolved in chloroform, was treated with a dilute solution of bromine in chloroform at room temperature until the bromine color was permanent. The mixture was allowed to stand several hours, the chloroform removed on the steam bath, and the product washed with water and dilute sodium bisulphite solution. After drying it was recrystallized from acetic acid. Dibromosphingosine sulphate forms a light gray crystalline powder, insoluble in alcohol and ether, soluble in acetic acid and chloroform.

0.1200 gm. substance gave 0.1811 gm. CO_2 and 0.0793 gm. H_2O.

	Calculated for $(C_{17}H_{33}NO_2Br_2)_2H_2SO_4$:	Found:
C	41.30	41.10
H	7.34	7.40

[3] Abderhalden, E., and Weil, A., *Ztschr. f. physiol. Chem.*, 1912, lxxviii, 150.

[4] Levene, P. A., and Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 127.

Dihydrosphingosine Sulphate.

This was prepared by the method already described by Levene and Jacobs. It was found in many experiments, especially if the ether contained alcohol, that the bulk of the dihydro base remained in the acetic acid-water solution with the palladium. It was isolated as follows: The ether layer was removed from the acetic acid and water. The ether was concentrated and the residue added to the dilute acid solution; this was then warmed and filtered to remove the palladium, and the filtrate treated with 10 per cent sulphuric acid as long as the sulphate separated. This was thoroughly cooled, filtered, and recrystallized from dilute alcohol (80 per cent). Dihydrosphingosine sulphate differs from that of sphingosine in that it is much less soluble in absolute alcohol and in chloroform.

Dihydrosphingosine Picrolonate.

This was prepared in the same way as the sphingosine picrolonate. It crystallizes in long, slender, yellow needles, which begin to soften at 110° and melt at 120–121°.

0.1317 gm. substance gave 0.2832 gm. CO_2 and 0.0966 gm. H_2O.

	Calculated for $C_{27}H_{46}N_8O_7$:	Found:
C	58.76	58.79
H	8.22	8.23

Dihydrosphingosine Picrate.

2 gm. of the dihydro base in 25 cc. of hot alcohol were treated with 25 cc. of a saturated alcoholic solution of picric acid, and hot water was added until the solution was turbid. The yellow precipitate was purified by recrystallization from dilute alcohol and finally by extraction with ether. The picrate forms pure yellow crystals, which melt at 88–89°.

0.1096 gm. substance gave 0.2150 gm. CO_2 and 0.0762 gm. H_2O.

	Calculated for $C_{23}H_{40}N_4O_9$:	Found:
C	53.44	53.50
H	7.80	7.78

Dihydrosphingosol (Trihydroxyheptyldecane) $C_{17}H_{33}(OH)_3$.

Dihydrosphingosine sulphate was dissolved in boiling acetic acid, cooled to room temperature, and gradually treated with slightly more than the theoretical amount of solid sodium nitrite. A concentrated aqueous solution of nitrite may also be used. After standing several hours the reaction product was concentrated to a small volume, poured into a large quantity of ice water, the precipitate filtered off and recrystallized several times from a small quantity of acetone, finally with the use of animal charcoal. *Dihydrosphingosol* is a colorless, crystalline solid, melting at 54–55° and easily soluble in all organic solvents.

	Calculated for $C_{17}H_{36}O_3$:	Found:
C	70.01	70.76
H	12.49	12.58

Hydroxyheptadecylamine, $C_{17}H_{34}(OH)NH_2$.

Hydroxyheptadecylamine was obtained as the reaction product of hydriodic acid upon an acetic acid solution of dihydrosphingosine sulphate either at 100° or at the boiling point of the mixture. The only influence the time of heating had was to decrease the yield, the maximum yield (which was always small) being obtained by 5 to 7 hours' heating. One experiment in which the heating was carried out for 20 hours gave the hydroxy-amine as the only basic reaction product. The other product of this reaction appears to be a hydrocarbon or alcohol, produced by the hydrolysis of the amino group.

10 gm. of dihydrosphingosine sulphate were dissolved in a mixture of 50 cc. of hydriodic acid and 200 cc. of glacial acetic acid, about 1 gm. of phosphonium iodide was added, and the solution heated in a boiling water bath for 5 to 7 hours. The solution was then concentrated in vacuum to a thick syrup, the residue washed with water and taken up in absolute alcohol. Alcoholic hydrochloric acid was added to the solution and the mixture gradually treated with metallic zinc until colorless. This colorless solution was concentrated in vacuum to a small volume and the salts, a mixture of the chloride and iodide, were precipitated with cold water. The free base was obtained by neutralizing the alcoholic

solution of the salts with sodium methylate, evaporating to dryness, and extracting with ether. This was changed into the sulphate by warming with dilute sulphuric acid, the crude product recrystallized from acetone, the non-basic part removed by washing with ether, and the sulphate finally recrystallized from absolute alcohol. It separates as long, slender, colorless needles, forming into rosettes, which melt at 206–208°. Many samples were analyzed, of which two are reported here:

0.1082 gm. substance gave 0.2528 gm. CO_2 and 0.1158 gm. H_2O.
0.1226 " " " 0.2893 " " " 0.1306 " "

	Calculated for $(C_{17}H_{37}O)_2H_2SO_4$:	Found:	
C	63.69	63.43	63.45
H	11.96	11.98	11.93

1.5 gm. of the sulphate were transformed into the free base. For this purpose it was dissolved in methyl alcohol. To the alcoholic solution was added a solution of barium hydroxide in methyl alcohol until it reacted alkaline to phenolphthalein. Acetone was then added to remove excess of barium, the filtrate concentrated in vacuum and recrystallized out of alcohol. The free base crystallized out in beautiful scales of the appearance of hydroxyheptadecylamine.[5] It melts at 85.5°.

The analysis of the substance was as follows:

0.1018 gm. substance gave 0.2756 gm. CO_2 and 0.1220 gm. H_2O.

	Calculated for $C_{17}H_{34}(OH)NH_2$:	Found:
C	75.3	74.12
H	13.68	13.47

The analysis shows the presence of a minimal quantity of dihydrosphingosine in the sample of the reduced base. This undoubtedly could be removed by repeated recrystallization.

[5] Levene, P. A., *Jour. Biol. Chem.*, 1915, xxiv, 79.

SPHINGOMYELIN. III.

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(Received for publication, November 30, 1915.)

Sphingomyelin was discovered by Thudichum.[2] This investigator established the principal distinction of this phosphatide from those of the lecithin group; namely, the absence of glycerol in its molecule. All other statements of the discoverer regarding sphingomyelin needed revision, since they were based on analysis of material that was not a pure phosphatide or pure phosphatides, but a mixture of these with cerebrosides. Rosenheim and Tebb[3] later improved the method of preparation of sphingomyelin, and had in their possession a much purer material. In a general way they accepted Thudichum's conclusions regarding the structure of the substance. Other investigators who, in course of their work on phosphatides, encountered the substance always dealt with grossly contaminated material.

In a previous communication[1] the writer reported a mode of procedure for preparing sphingomyelin free from cerebrosides, and also the conditions for the hydrolysis of the substance. The following substances were then obtained: (1) phosphoric acid; (2) two fatty acids, cerebronic and lignoceric; (3) three basic substances, choline, sphingosine, and a base of the composition $C_{17}H_{35}NO$. Sphingosine had not been isolated in pure form, and the composition of the third base was rather suggested than definitely demonstrated. Since that publication the work has been continued and extended to the sphingomyelin obtained from other organs; namely, kidney, liver, and the yolk of hen's

[1] Levene, P. A., *Jour. Biol. Chem.*, 1913, xv, 153; 1914, xviii, 453.

[2] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884, 105.

[3] Rosenheim, O., and Tebb, M. C., *Quart. Jour. Physiol.*, 1908, i, 297; *Jour. Physiol.*, 1910, xli, p. i.

egg. The method of preparation of the substance was slightly modified with the result that the sphingomyelin employed in the present work was entirely free from contaminating cerebrin.

The elementary composition and the specific optical rotation of the new material were quite constant. Sphingomyelin is microcrystalline in nature.

Employing the old method of hydrolysis the products obtained from the new material differed from those recorded in the earlier work only in the point of the acids. Cerebronic and lignoceric were the two previously recorded. Cerebronic acid could not be detected in the course of the present work. In the older material it undoubtedly originated in the adhering cerebrin. Lignoceric acid was identified beyond doubt, and with it was demonstrated the presence of another acid, of lower molecular weight. From the present data it seems to be a hydroxy acid. The mixed acids obtained on hydrolysis of sphingomyelin contain at least 50 per cent of the new acid. However, it was not prepared in a state of satisfactory purity through any of the conventional methods of fractionation, either through fractional crystallization from organic solvents, through the sodium, lithium, or magnesium salts, or through fractional distillation of the esters. It is hoped, however, that with a larger quantity of material on hand the difficulty will be overcome.

For the bases the old method of hydrolysis gave results which in every way substantiated the older conclusions. Again choline, sphingosine, and a base $C_{17}H_{35}NO$ were identified. The assumed composition of $C_{17}H_{35}NO$ was demonstrated to be the correct one; and after reduction with hydrogen and palladium it was shown to possess the structure of hydroxyheptadecylamine $C_{17}H_{34}(OH).NH_2$. The latter base gave up, in 10 minutes, all its nitrogen in the form of nitrogen gas on treatment with nitrous acid in the Van Slyke apparatus, and, further, it formed a diacetyl derivative. The reduced base will be referred to as sphingine. In the free state it crystallizes in beautiful plates that have the appearance of cholesterol. However, in the course of the work sufficient evidence accumulated for the belief that the base was a secondary product formed from sphingosine. In the process of hydrolysis of sphingomyelin with alcohol and water sphingosine loses a molecule of water, giving rise to a base of the nature of anhydrosphingosine,

$$C_{12}H_{25}CH=CHCH=C(OH)CH_2NH_2.$$

The position of the additional double bond in this graphic formula is arbitrary. Owing to the great solubility of the free base or of its sulphate in organic solvents it generally escaped detection, but it always contaminated the sphingosine prepared from sphingomyelin. It was perhaps due to this impurity that Thudichum never succeeded in isolating pure sphingosine from sphingomyelin. He referred to the base isolated by him as an impure sphingosine or a homologue of sphingosine.

In our own work the base escaped detection for a long time. Only after the mixed bases were reduced by hydrogen in the presence of colloidal palladium could their separation be accomplished; namely, through the fractional crystallization of their sulphates from a solution of equal parts of chloroform and amyl alcohol.

The reasons for the belief in the secondary origin of anhydrosphingosine are the following: when the cleavage of sphingomyelin was accomplished through hydrolysis in sealed tubes with 3 per cent sulphuric acid solution, anhydrosphingosine could not be detected, and sphingosine was easily isolated in pure condition. On the other hand, when a sample of pure cerebrin—corresponding to Thierfelder's cerebron—was hydrolyzed by the combined method and the bases were subsequently reduced, in place of dihydrosphingosine, pure sphingine was obtained. There should be recorded here another difficulty in identifying sphingosine. The sulphate which generally serves for identification of the base on recrystallization easily changes its composition, a part of the neutral salts changing into either the basic or the acid salt. The same applies to sphingine. It was therefore found necessary to purify sphingosine and more so dihydrosphingosine and sphingine by converting them into the free bases; these may then be reconverted into the sulphate if desired.

Thus, for the present, sphingomyelin was found to yield on hydrolysis phosphoric acid, two fatty acids, and two bases, choline and sphingosine.

Thudichum and Rosenheim and Tebb accepted in the molecule of sphingomyelin besides the bases and the acids also an alcohol. This view does not agree with the analytical finding of the present work; namely, the cleavage of our material yielded 34 per cent of

its weight in the form of sphingosine and 43 per cent in the form of the organic acids. These values leave no room in the molecule of the phosphatide for the alcohol sphingol, and, on the other hand, they agree with the assumption that a monophosphatide of the sphingomyelin type is composed of single equivalent proportions of the components isolated by us.

Hence, for the present one is justified in assuming for a phosphatide of this type the structural expression,

$$O=P\begin{cases}O-C_{17}H_{32}(OH)NH.COC_{23}H_{47}\text{ (or another acid radicle)}\\ OH\\ O.C_5H_{13}N(OH)\end{cases}$$

This assumption is further justified by a chance discovery of an intermediary product. This was obtained on hydrolysis of a sample of kidney sphingomyelin. It possessed the following properties: it contained nitrogen, gave no nitrogen gas on treatment with nitrous acid, did not form salts with mineral acids, and had the elementary composition of lignoceryl sphingine, $C_{17}H_{34}(OH)NH.CO.C_{23}H_{47}$. Lack of material prevented hydrolysis of the substance.

Whether sphingomyelin is a mixture of two phosphatides of the described type, or a diphosphatide, can for the present not be stated with certainty. This question as well as the question of the structure of the second fatty acid is under investigation.

Regarding the relation of the sphingomyelins derived from different organs, one is inclined to believe in their identity. If ultimately it will be demonstrated that sphingomyelin is a mixture of two monophosphatides, then it may appear that their proportion varies somewhat in the material obtained from different organs. The following table of the elementary analysis and of the physical properties of samples derived from different sources brings out best the justification for the expressed belief.

Source.	C	H	N	P	$[\alpha]_D$	Sphingosine.	Acids.
						per cent	*per cent*
Brain	66.59	11.26	3.78	3.99	+8.20 +7.53	34.10	43.00
Kidney	64.80	11.41	3.50	3.82	+8.73	32.10	49.00
Liver	64.47	11.57	3.41	3.81	+7.61	32.14	41.70
Egg yolk	65.56	11.68	3.84	4.22	+7.54	33.70	43.40

EXPERIMENTAL PART.

I. Brain Sphingomyelin.[4]

The crude sphingomyelin was prepared according to the directions given in the previous communication. This substance was dissolved in five parts of ligroin and one part of alcohol. Alcohol was then added as long as a precipitate formed. The filtrate was allowed to stand over night at 0°C. and again filtered. The final filtrate was concentrated under diminished pressure and poured into acetone.

The material obtained in this manner was further purified by recrystallization out of a solution in equal parts of pyridine and chloroform. This was carried out in several stages. Five recrystallizations were made at room temperature; these were followed by recrystallizations at 30°, and finally at 37°. The recrystallizations were continued until the substance gave a negative orcin test in the presence of a trace of copper acetate.

Elementary Composition of Sphingomyelin.

I. 0.2835 gm. substance, dried in a vacuum desiccator at the temperature of boiling water, gave 0.0406 gm. $Mg_2P_2O_7$.

0.1890 gm. material required for neutralization 5.1 cc. $\frac{N}{10}$ H_2SO_4.

0.1000 gm. substance gave 0.2430 gm. CO_2, 0.1006 gm. H_2O, and 0.0104 gm. ash.

The optical rotation of the substance dissolved in equal parts of chloroform and methyl alcohol was as follows:

$$[\alpha]_D^{25} = \frac{+0.34^\circ \times 3.6220}{0.5 \quad \times 0.3000} = +8.20^\circ$$

II. 0.3000 gm. substance, dried as Sample I, gave 0.0428 gm. $Mg_2P_2O_7$.

0.2000 gm. substance required 5.2 cc. $\frac{N}{10}$ H_2SO_4.

0.0996 gm. substance gave 0.2438 gm. CO_2 and 0.1012 gm. H_2O.

The optical rotation in chloroform-methyl alcohol solution was:

$$[\alpha]_D^{25} = \frac{+0.34^\circ \times 3.6198}{0.5 \quad \times 0.3000} = +8.20^\circ$$

III. 0.4000 gm. substance, dried as Sample I, gave 0.0564 gm. $Mg_2P_2O_7$.

0.2000 gm. substance required for neutralization 5.1 cc. $\frac{N}{10}$ H_2SO_4.

0.0990 gm. substance gave 0.2402 gm. CO_2 and 0.1044 gm. H_2O.

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[4] The mode of procedure described for the brain tissue was followed in the work on the other organs.

The optical rotation in chloroform-methyl alcohol solution was:

$$[\alpha]_D^{25} = \frac{+0.31^\circ \times 3.6460}{0.5 \times 0.3000} = +7.53^\circ$$

IV. 0.3000 gm. substance, dried as Sample I, gave 0.0434 gm. $Mg_2P_2O_7$.
0.2000 gm. substance required for neutralization 5.2 cc. $\frac{N}{10}$ H_2SO_4.
0.0990 gm. substance gave 0.2410 gm. CO_2 and 0.0998 gm. H_2O.
The optical rotation in the same solution as the other samples was:

$$[\alpha]_D^{25} = \frac{+0.31^\circ \times 3.6404}{0.5 \times 0.3000} = +7.52^\circ$$

Table of Analysis.

	C	H	N	P
I	66.27	11.26	3.78	3.99
II	66.81	11.30	3.64	3.97
III	66.65	11.59	3.57	3.92
IV	66.45	11.21	3.64	4.02
Average	66.59	11.34	3.66	3.98

The variations in the elementary composition of the various samples are slight, especially when the size of the molecule and the physical properties of the substance are taken into consideration.

Methods of Hydrolysis.

Two methods of hydrolysis were employed in the course of the work.

One was described in the earlier paper and was carried out in two stages. In the first the substance was heated with barium hydroxide, in the second with hydrochloric acid. This method will be referred to as the *combined method.*

The second method was carried out in the following way. One part of the substance and fifteen parts by weight of 3 per cent sulphuric acid were heated in sealed tubes for 24 hours at a temperature of 100–105°. From time to time the tubes were agitated. On cooling, the fatty acids and sphingosine sulphate separated as a soft cake which could be readily removed by filtration and washed.

Separation of the Product of Hydrolysis into Three Fractions.

Choline Fraction.—This was contained in the aqueous part of the products of hydrolysis. The analysis of this fraction was carried out in the manner described in the previous paper.

Acid Fraction.—In all experiments the acids were separated from the bases by virtue of the nearly complete insolubility of the barium salts of the higher fatty acids in acetone. The free bases, on the other hand, are soluble in the same solvent.

Sphingosine Fraction.—This was present in the acetone filtrate from the soaps.

For convenience of discussion of the results obtained in this work the mode of procedure in the two types of hydrolysis will be reiterated.

Combined Hydrolysis.—The water-insoluble part of the products of hydrolysis was dissolved in a minimum volume of methyl alcohol to which was added in excess a solution of barium hydroxide in methyl alcohol; to complete the separation of the barium soaps an excess of acetone was added. The precipitate consisted of the soaps, and the filtrate contained the ethyl esters of the fatty acids and the water-insoluble bases.

In order to saponify the esters the acetone solution was concentrated under diminished pressure and the residue dissolved in methyl alcohol. To this solution, barium hydroxide in methyl alcohol was added in excess and the product heated on the water bath with a reflux condenser for 6 hours. From the reaction product the separation of the barium soaps was completed by the addition of a considerable quantity of acetone. To further purify the acid fraction the acids were liberated from the barium soaps, and reconverted into the barium soaps by the barium and acetone procedure.

In order to purify the basic fraction the acetone solution was concentrated *in vacuo*, the residue dissolved in methyl alcohol, and the barium hydroxide and acetone procedure was repeated. As a rule, the entire process was repeated three times. The careful treatment of this fraction was found essential. The presence of impurities, particularly of inorganic nature, interferes considerably with the subsequent reduction of the bases with hydrogen.

Sulphuric Acid Hydrolysis.—The water-insoluble products of hydrolysis under these conditions consisted only of the acids and of sphingosine; there was no need for the stage which aimed to saponify the esters. Hence the reaction product was dissolved in methyl alcohol and treated with barium hydroxide and acetone, as described above.

Quantitative Hydrolysis.—These experiments aimed to determine the proportion of the constituents, and their general elementary composition, without attempting to determine the exact nature of the acids and the bases. Their purpose was to ascertain whether the substances isolated in the course of this work make up the entire molecule of sphingomyelin. This hydrolysis was carried out by means of 3 per cent sulphuric acid. 2 gm. of the substance were used for each experiment.

Analysis of Fatty Acids.

Liberation and Purification of Fatty Acids.—The mode of liberation of the fatty acids and of their purification remained the same in all experiments. The barium soaps were suspended in aqueous 10 per cent hydrochloric acid, and allowed to stand on the water bath over night. On cooling, a solid mass formed consisting of the free acids and some adhering barium chloride. The mass was filtered and taken up in hot acetone, which separated the acids from the mineral impurities. The acetone solution was then concentrated *in vacuo* and the residue was again taken up in acetone. The operation was repeated until the residue completely dissolved in acetone, leaving no filterable residue. The acetone was then removed by distillation and the residue concentrated on the water bath to constant weight.

The fatty acids obtained in this manner served for the determination of the elementary composition and of the molecular weight of the mixed acids. Whenever there was still present a slight mineral impurity, the acids were further purified by transferring them into lead salts. The mode of procedure was the following: The acids were dissolved as rapidly as possible in hot methyl alcohol, and the solution was treated with a slight excess of lead acetate dissolved in methyl alcohol. The addition of a few drops of ammonia completed the precipitation. The lead salts were

washed with water and with acetone, and then suspended in ether and allowed to stand over night. Finally the lead salts were dissolved in hot toluene and decomposed by means of hydrogen sulphide.

Practically all samples of which the molecular weights are recorded were prepared by this procedure.

Separation of the Fatty Acids.—A general method for the separation of fatty acids of sphingomyelin has as yet not been devised. Of the many employed in course of the present work only those will be reported which helped to bring out two points: first, that one of the acids making up the molecule of the phosphatide is lignoceric; and second, that the other is of a lower molecular weight and appears to be a hydroxy acid.

Experiment I.—90.0 gm. of sphingomyelin were hydrolyzed by the combined method. The acids were isolated in two fractions: the first as the barium salts of the unesterified acids; and the second as the barium salts of the saponified esters.

Analysis of the purified acids of the first fraction:

0.1000 gm. substance gave 0.2769 gm. CO_2 and 0.1144 gm. H_2O.

1.0000 " " required for neutralization 6.4 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	75.49
H	13.20	12.71
Mol. wt	368	312

Acids of the second fraction:

0.1000 gm. substance gave 0.2772 gm. CO_2 and 0.1158 gm. H_2O.

1.0000 " " required for neutralization 6.4 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	75.59
H	13.20	12.95
Mol. wt	368	312

The acids were then combined and repeatedly recrystallized at 25°C. out of a large volume of acetone. Finally the most insoluble fraction had a melting point of 80–82°.[5]

1.0000 gm. of this substance required for neutralization 5.6 cc. $\frac{N}{2}$ alkali; hence mol. wt. = 358.

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[5] All melting points were determined under exactly the same condition; namely, in a sulphuric acid bath provided with a stirring arrangement. The rate of heating was 6 to 7 seconds per 1°C.

Experiment II.—30.0 gm. substance were hydrolyzed in sealed tubes. The fatty acids were immediately transformed into the methyl esters. The esters were distilled under a pressure of 0.3 mm. Two fractions were obtained, the first distilling up to 155°, the second distilling up to 200°.

The esters of the second fraction had a melting point of 56–57°C., and the following composition:

0.1010 gm. substance gave 0.2906 gm. CO_2 and 0.1190 gm. H_2O.

	Calculated for $C_{24}H_{47}O_2CH_3$:	Found:
C	78.53	78.47
H	13.00	13.19

The ester was saponified and the resulting acid had a melting point of 81°C.

Experiment III.—100.0 gm. of sphingomyelin were decomposed by the combined method. The acids were purified by the lead method and fractionated from acetone. The fraction of higher solubility had the following composition:

0.1025 gm. substance gave 0.2758 gm. CO_2 and 0.1096 gm. H_2O.

C = 73.46; H = 11.98.

The substance, again recrystallized out of acetone, had the following composition:

0.1054 gm. substance gave 0.2778 gm. CO_2 and 0.1166 gm. H_2O.

0.3000 " " required 2.0 cc. $\frac{N}{2}$ alkali for neutralization.

The substance had a melting point of 68–69°C.

	Calculated for $C_{18}H_{36}O_2$:	Found:
C	71.92	71.89
H	12.10	12.38
Mol. wt.	300	300

The substance was converted into the ethyl ester and the ester recrystallized out of alcohol. The middle fraction had the following composition:

0.0844 gm. substance gave 0.2246 gm. CO_2 and 0.0916 gm. H_2O.

	Calculated for $C_{18}H_{35}O_2C_2H_5$:	Found:
C	73.07	72.58
H	12.27	12.15

This acid possessed the elementary composition and the molecular weight of hydroxystearic acid. However, the low melting point of the acid warned us against hasty conclusions. A corresponding fraction from the kidney sphingomyelin possessed a still lower molecular weight. Hence decision as to the actual composition of the second acid will be deferred until we accumulate more material which will permit further purification of the acid.

Bases.

The mode of procedure in the analysis of the sphingosine fraction differed in the earlier and later experiments. Originally the fraction was treated with alcoholic sulphuric acid to form the sulphates of the bases. These were then reduced with hydrogen gas in the presence of colloidal palladium after Paal. In later experiments it was found more advantageous to reduce the entire fraction prior to the formation of the sulphates. In order to facilitate reduction, the bases were dissolved in ether and washed with water in a separatory funnel. To the ethereal solution of the bases some acetic acid was added and the solution then reduced with hydrogen.

In order to remove the palladium completely the product of reduction was warmed on the water bath with an equal volume of acetone. The filtrate was concentrated to remove all the acetone and acetic acid. The residue was then dissolved in an equal volume of alcohol and an alcoholic solution of sulphuric acid was added until the mixture reacted faintly acid to Congo red. Care must be taken not to overadd sulphuric acid, as the sulphate is quite soluble in an excess of the acid.

Analysis of the bases obtained in this manner gave the following results:

I. Combined method of hydrolysis.
0.1024 gm. substance gave 0.2772 gm. CO_2 and 0.0954 gm. H_2O.
C = 62.37; H = 10.35.

II. Combined method of hydrolysis.
0.1012 gm. substance gave 0.2336 gm. CO_2 and 0.0970 gm. H_2O.
C = 62.79; H = 10.80.

A great many samples of similar composition were analyzed, and material of such composition served for the isolation of hydroxyheptadecylamine.

Hydroxyheptadecylamine (Sphingine),[6] $C_{17}H_{34}(OH)NH_2$.—This base was obtained by fractional crystallization of the mixed sulphates from a solution of chloroform in amyl alcohol. The first recrystallizations were carried out from a solution in one part of chloroform and three parts of alcohol. The concentration of the chloroform was progressively increased and the final re-

[6] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1915, xxiv, 67.

crystallizations were made out of equal parts of the solvents, first at room temperature (20–25°C.) and finally at 35°.

The material obtained in this manner consisted of microscopic prismatic needles, and had the macroscopic appearance of glistening scales.

The composition of this crystalline sulphate was not constant for individual samples, nor for successive recrystallizations of the same sample, although a sufficient number gave on analysis values corresponding to the neutral sulphate of sphingine.

I. 0.1020 gm. substance gave 0.2326 gm. CO_2 and 0.1034 gm. H_2O.
0.2000 " " required for neutralization 5.8 cc. $\frac{N}{10}$ acid.
0.1000 " " gave 0.0366 gm. $BaSO_4$.
II. 0.1012 " " " 0.2344 gm. CO_2 and 0.0990 gm. H_2O.
0.2000 " " required for neutralization 5.3 cc. $\frac{N}{10}$ acid.
0.3000 " " gave 0.1126 gm. $BaSO_4$.
III. 0.1020 " " " 0.2376 gm. CO_2 and 0.1116 gm. H_2O.

	Calculated for $[C_{17}H_{37}NO]_2H_2SO_4$:	Found: I.	II.	III.
C	63.80	63.43	63.43	63.77
H	11.85	11.57	11.01	12.10
N	4.37	4.06	4.06	
S	5.00	5.03	5.15	

These samples on further recrystallization gave values for carbon varying from 64.5 to 66.0, and for hydrogen from 11.5 to 12.5. However, when converted into the free base they generally had the uniform composition of sphingine.

Free Sphingine.—For the preparation of the free base the sulphate was treated in the same manner as sphingosine sulphate. The base crystallized out of a minimal quantity of petrolic ether in the form of plates resembling cholesterol. The substance had a melting point of 83.5°, and had the following composition:

0.1046 gm. substance gave 0.2844 gm. CO_2 and 0.1280 gm. H_2O.
0.1056 " " " 0.2868 " " " 0.1238 " "

	Calculated for $C_{17}H_{37}NO$:	Found:	
C	75.30	74.90	75.35
H	13.65	13.82	13.35

In a 10 per cent solution of sulphuric acid in alcohol the substance had the following rotation:

$$[\alpha]_D^{25} = \frac{-0.34^\circ \times 1.7660}{1 \quad \times 0.1000} = -6.00^\circ$$

Diacetylhydroxyheptadecylamine.—0.6000 gm. of the free base was heated with acetic anhydride for 1 hour under a reflux condenser. On cooling the acetyl derivative crystallized in the form of long needles. The substance was recrystallized out of acetone. It melts at 109.5°C. (corrected).

0.1050 gm. substance gave 0.2730 gm. CO_2 and 0.1108 gm. H_2O.
0.1000 " " required for neutralization 2.71 $\frac{N}{10}$ cc. acid.

	Calculated for $C_{17}H_{35}NO(CH_3CO)_2$:	Found:
C	70.90	70.90
H	11.55	11.80
N	3.97	3.79

In a solution in equal parts of chloroform and methyl alcohol the substance gave the following rotation:

$$[\alpha]_D^{25} = \frac{+0.44^\circ \times 2.3234}{1 \quad \times 0.0500} = +20.44^\circ$$

Dihydrosphingosine (Dihydroxyheptadecylamine).—For the identification of sphingosine, use was made of the hydrolysis by means of 3 per cent aqueous sulphuric acid. The basic fraction was reduced with hydrogen in the presence of colloidal palladium. Since the purification by means of repeated recrystallization out of alcohol containing small quantities of sulphuric acid gave unsatisfactory results, it was considered essential to analyze the free base.

Analysis of the sulphate:

0.1040 gm. substance gave 0.2324 gm. CO_2 and 0.1070 gm. H_2O.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$:	Found:
C	60.61	60.94
H	11.38	11.51

The free base was obtained by the process employed in course of this work.

0.1023 gm. substance gave 0.2664 gm. CO_2 and 0.1204 gm. H_2O.

	Calculated for $C_{17}H_{37}NO_2$:	Found:
C	71.07	71.02
H	12.89	13.17

Hydroxyheptadecylamine from Cerebrin.—80.0 gm. of cerebrin, corresponding to Thierfelder's cerebron, were hydrolyzed by the

combined method, and the mode of procedure employed in the hydrolysis of sphingomyelin was followed closely. The free base obtained in this manner had the physical properties of sphingine and the following composition:

0.1042 gm. substance gave 0.2874 gm. CO_2 and 0.1232 gm. H_2O.

	Calculated for $C_{17}H_{37}NO$:	Found:
C	75.30	75.12
H	13.65	13.23

Choline.—Choline was isolated from the aqueous part of the product of hydrolysis. When the hydrolysis was accomplished by means of aqueous sulphuric acid, the aqueous filtrate was treated with barium carbonate and the filtrate from barium sulphate was concentrated to dryness under diminished pressure. The residue was taken up in alcohol, filtered, and concentrated, and the operation was repeated several times. The final product was acidulated with hydrochloric acid and treated with an alcoholic solution of platinic chloride. The platinic salt was recrystallized out of water.

When barium hydroxide was used for hydrolysis the aqueous part of the product of hydrolysis was saturated with carbon dioxide gas, and the filtrate from barium carbonate was used for the preparation of the chloroplatinic salt of choline. The solution apparently contained no other bases than choline, since on treatment with nitrous acid in Van Slyke's apparatus it did not give off nitrogen gas.

0.1018 gm. substance gave 0.0732 gm. CO_2, 0.0430 gm. H_2O, and 0.0316 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.60
H	4.58	4.69
Pt	31.65	31.05

Quantitative Hydrolysis.

In order to obtain an estimate of the fatty acids and of sphingosine in sphingomyelin, hydrolysis was carried out on 2.0 gm. of the substance in a sealed tube with a 3 per cent sulphuric

acid solution. The mode of procedure was the same as that carried out on larger quantities of material.

The empirical data obtained in this work will be compared with the theoretical for the monophosphatide containing lignoceric acid in its molecule.

	Calculated for $C_{46}H_{93}N_2O_7P$:	Found:
Sphingosine	35.56	34.10
Acids	40.58	43.00

The crude sphingosine fraction contained 4.5 per cent nitrogen. It was transformed into a sulphate of the following composition:

0.1012 gm. substance gave 0.2218 gm. CO_2 and 0.0928 gm. H_2O.

	Calculated for $(C_{17}H_{33}NO_2)_2H_2SO_4$:	Found:
C	61.08	60.97
H	10.78	10.47

Analysis of the acid fraction:

0.1022 gm. substance gave 0.2876 gm. CO_2 and 0.1188 gm. H_2O.

1.0000 " " was titrated with $\frac{N}{2}$ alkali, and required for neutralization 6.3 cc.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	76.75
H	13.20	13.00
Mol. wt.	368	317

II. Beef Kidney Sphingomyelin.

A phosphatide of this group was first discovered in the extracts from beef kidney by E. K. Dunham,[7] and was named by him carnaubon. Later MacLean[8] questioned the correctness of Dunham's views by showing that carnaubon could be further purified, and still later Rosenheim and MacLean[9] have demonstrated that carnaubon contained an admixture of galactosides, and that the acid assumed by Dunham to be carnaubic had the physical properties of lignoceric acid. However, Rosenheim and MacLean have not isolated the pure sphingomyelin.

[7] Dunham, E. K., *Proc. Soc. Exper. Biol. and Med.*, 1904–05, ii, 63; *Jour. Biol. Chem.*, 1908, iv, 297. Dunham, E. K., and Jacobson, C. A., *Z. f. physiol. Chem.*, 1910, lxiv, 302.

[8] MacLean, H., *Biochem. Jour.*, 1912, vi, 333.

[9] Rosenheim, O., and MacLean, H., *Biochem. Jour.*, 1915, ix, 103.

The present investigation was in progress at the time of the publication of their results.

Preparation and Composition.—All the details found necessary for the preparation of brain sphingomyelin were applied for the isolation of the substance from the kidney tissue. Several samples were analyzed and all seemed to contain a slightly lower proportion of carbon. This was possibly due to the fact that the material from the kidney contained a higher proportion of the phosphatide with the fatty acid of a molecular weight lower than that of lignoceric.

I. 0.1032 gm. substance gave 0.2412 gm. CO_2 and 0.1052 gm. H_2O.
0.3000 " " required for neutralization 7.5 cc. $\frac{N}{10}$ acid.
0.4000 " " gave 0.0548 gm. $Mg_2P_2O_7$.
II. 0.0990 " " " 0.2330 gm. CO_2 and 0.1042 gm. H_2O.
0.2000 " " required for neutralization 4.75 cc. $\frac{N}{10}$ acid.
0.3000 " " gave 0.0404 gm. $Mg_2P_2O_7$.

	C	H	N	P
I	64.80	11.41	3.50	3.82
II	64.53	11.83	3.52	3.96

In a solution of methyl alcohol and chloroform Sample I gave the following optical rotation:

$$[\alpha]_D^{36} = \frac{+0.36^\circ \times 3.6510}{0.5 \times 0.3000} = +8.73^\circ$$

Quantitative Hydrolysis.

2.0 gm. of Sample I, hydrolyzed for 24 hours in a sealed tube with 3 per cent sulphuric acid, gave 32.1 per cent of its weight in the sphingosine fraction and 49.0 per cent in the fraction of the fatty acid.

Choline Fraction.—This fraction was analyzed in the same manner as the corresponding fraction from brain sphingomyelin. The base was identified as the salt of chloroplatinic acid. The salt was recrystallized out of water.

0.1048 gm. substance gave 0.0752 gm. CO_2, 0.0434 gm. H_2O, and 0.0334 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.58
H	4.58	4.63
Pt	31.65	31.87

Acid Fraction.—Here also final results on the separation of the two acids have not yet been obtained. The mixed acid showed approximately the same composition and molecular weight as the corresponding material from the brain sphingomyelin.

Experiment I.—Acid hydrolysis. The acid fraction was purified through the lead salts.

I. 0.1020 gm. substance gave 0.2830 gm. CO_2 and 0.1135 gm. H_2O.
II. 0.1014 " " " 0.2846 " " " 0.1128 " "
0.4062 " mixed acids required for neutralization 2.56 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_2$:	Found: I.	II.
C	78.20	75.96	76.53
H	13.20	12.49	12.45
Mol. wt	368	312	315

Experiment II.—The most convenient method for obtaining lignoceric acid was through the process of alcoholysis. 9.0 gm. of sphingomyelin in a solution of 4.0 cc. of sulphuric acid in 200 cc. of methyl alcohol were heated under a reflux condenser on the water bath for 6 hours. On cooling an ester separated out in bright scales. Recrystallized out of acetone, the substance contained: C, 74.11, and H, 12.13. The substance contained 1.17 per cent of ash. It was distilled at a pressure of 0.4 mm. The temperature of distillation was allowed to rise to 200°C. The distillate was recrystallized out of acetone. The substance had a melting point of 56–57° and the following composition:

0.1020 gm. substance gave 0.2934 gm. CO_2 and 0.1200 gm. H_2O.

	Calculated for $C_{24}H_{47}O_2CH_3$:	Found:
C	78.53	78.44
H	13.09	13.17

The acid of the lower molecular weight has not yet been isolated in a sufficient degree of purity. The best results were obtained by fractionation from acetone. In two experiments a substance was obtained of apparently the same composition and molecular weight. But here again decision regarding its true composition will have to be deferred until a larger quantity of the acid is on hand. In both experiments the acids were purified through the lead salts prior to fractionation. The two results will be described under Experiment III.

Experiment III.—The mixed acids were recrystallized out of acetone at 0°, the mother liquor was concentrated and again taken up in hot acetone. The final substance had the following composition:

0.0952 gm. substance gave 0.2558 gm. CO_2 and 0.1152 gm. H_2O.
C=69.76; H=10.65.

A second sample, obtained in the same manner, had the following composition:

0.1042 gm. substance gave 0.2670 gm. CO_2 and 0.1032 gm. H_2O.
C=69.88; H=11.09.

Sphingosine Fraction.—The mixed bases obtained by the combined method of hydrolysis, following exactly the mode of procedure described above, gave results approximating those obtained on the brain sphingomyelin. The product consisted of long needles.

0.1046 gm. substance gave 0.2400 gm. CO_2 and 0.1092 gm. H_2O.
C=62.79; H=11.69.

This substance was further fractionated from amyl alcohol and chloroform and finally transformed into the free base. This had the appearance of sphingine and the following composition:

0.1030 gm. substance gave 0.2756 gm. CO_2 and 0.1222 gm. H_2O.

	Calculated for $C_{17}H_{37}NO$:	Calculated for $C_{17}H_{37}NO_2$:	Found:
C	75.30	71.07	73.12
H	13.65	12.89	13.30

Pure sphingosine was more accessible through the sulphuric acid hydrolysis. The substance was reduced with hydrogen prior to conversion into the sulphate.

I. 0.0992 gm. substance gave 0.2158 gm. CO_2 and 0.1042 gm. H_2O.
0.1410 " " required for neutralization 4.25 cc. $\frac{N}{10}$ acid.
II. 0.1048 " " gave 0.2340 gm. CO_2 and 0.1032 gm. H_2O.
0.1500 " " required for neutralization 4.34 cc. $\frac{N}{10}$ acid.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$:	Found: I.	Found: II.
C	60.61	60.67	60.90
H	11.38	11.35	11.02
N	4.16	4.23	4.05

Lignoceryl Sphingine.

The substance was obtained in the course of a combined hydrolysis of 100 gm. of Sample I (page 84). The basic fraction was reduced with hydrogen in the presence of palladium after Paal.

The reaction product was warmed on the water bath and filtered. The filtrate was concentrated nearly to dryness on the water bath. The residue was dissolved in 400 cc. of hot alcohol and filtered hot. While the solution was still warm a crystalline precipitate formed. This was immediately filtered, and recrystallized out of alcohol. It crystallized in the form of rosettes composed of needles.

The substance gave no nitrogen gas on treatment with nitrous acid according to Van Slyke.

0.1018 gm. substance gave 0.2874 gm. CO_2 and 0.1173 gm. H_2O.
0.1473 " " neutralized 2.72 cc. $\frac{N}{10}$ acid.

	Calculated for $C_{17}H_{34}(OH)NH.C_{24}H_{47}O$:	Found:
C	79.39	79.60
H	13.36	13.32
N	2.26	2.59

Lack of material prevented further work on this substance.

III. Liver Sphingomyelin.

No phosphatide of this type has been previously obtained from this organ. The substance here analyzed was prepared in the usual way. The crude sphingomyelin required a great many recrystallizations from pyridine and chloroform. Finally it reacted towards orcin in the same manner as sphingomyelin of other origin.

0.1067 gm. substance gave 0.2500 gm. CO_2 and 0.1088 gm. H_2O.
0.3000 " " required for neutralization 7.3 cc. $\frac{N}{10}$ acid.
0.4000 " " gave 0.0548 gm. $Mg_2P_2O_7$.

C	H	N	P
64.47	11.57	3.41	3.81

In a solution of equal parts of methyl alcohol and chloroform the substance had the following rotation:

$$[\alpha]_D^{32} = \frac{+0.31° \times 3.6490}{0.5 \times 0.3000} = +7.61°$$

Quantitative Hydrolysis.

2.0 gm. substance, hydrolyzed with 3 per cent H_2SO_4, gave 32.14 per cent of its weight in the form of sphingosine and 41.70 per cent in the form of acids.

	Calculated for $C_{46}H_{96}N_2O_7P$:	Found:
Sphingosine	35.56	32.14
Acids	40.58	41.70

Choline.—This base was obtained in the usual way. The salt of chloroplatinic acid was recrystallized out of water.

0.1000 gm. substance gave 0.0718 gm. CO_2, 0.0428 gm. H_2O, and 0.0312 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.58
H	4.58	4.79
Pt	31.65	31.20

Sphingosine.—The substance was obtained in the usual way. It was analyzed in the form of the sulphate.

0.1075 gm. substance gave 0.2282 gm. CO_2 and 0.0952 gm. H_2O.

	Calculated for $(C_{17}H_{35}NO_2)_2H_2SO_4$:	Found:
C	61.08	60.78
H	10.78	10.40

Acids.—The mixed acids were obtained by hydrolysis with 3 per cent sulphuric acid in sealed tubes and had the following composition:

0.1075 gm. substance gave 0.2956 gm. CO_2 and 0.1206 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	75.00
H	13.20	12.60

IV. Egg Yolk Sphingomyelin.

A phosphatide approximating in composition that of Thudichum's sphingomyelin was described by Stern and Thierfelder.[10] The material described here was prepared by the same mode of procedure used in the work on sphingomyelin from other sources and had the same microscopic appearance. It gave a negative test on boiling with orcin in the presence of copper salts.

0.0993 gm. substance gave 0.2354 gm CO_2 and 0.1030 gm. H_2O.
0.4000 " " gave 0.0606 gm. $Mg_2P_2O_7$.
0.3000 " " required for neutralization 8.3 cc. $\frac{N}{10}$ acid.

C	H	N	P
65.00	11.68	3.84	4.22

[10] Stern, M., and Thierfelder, H., *Z. f. physiol. Chem.*, 1907, liii, 370.

In a solution of equal parts of methyl alcohol and chloroform the substance had the following rotation:

$$[\alpha]_D^{25} = \frac{+0.31° \times 3.6500}{0.5 \times 0.3000} = +7.54°$$

Quantitative Hydrolysis.

2 0 gm. substance, hydrolyzed with 3 per cent sulphuric acid, gave in the acid fraction 43.14 per cent of its weight and 33.70 per cent in the sphingosine fraction.

	Calculated for $C_{46}H_{92}N_2O_7P$:	Found:
Sphingosine	35.56	33.70
Acids	40.58	43.14

Choline.—This was obtained in the usual way and recrystallized out of water.

0.1030 gm. substance gave 0.0734 gm. CO_2, 0.0460 gm. H_2O, and 0.0324 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.43
H	4.58	4.88
Pt	31.65	31.46

Sphingosine Fraction.—This was obtained in the usual way and reduced to dihydrosphingosine. The value obtained for the carbon slightly exceeds the theoretical requirement, and the evidence is accepted as satisfactory only in the light of the experience gained with the substance obtained from the brain and from the kidneys.

0.1039 gm. substance gave 0.2348 gm. CO_2 and 0.1054 gm. H_2O.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$:	Found:
C	60.61	61.75
H	11.38	11.37

Analysis of Mixed Acids.—The acids were obtained by hydrolysis with 3 per cent sulphuric acid and had the same composition as the acids obtained from sphingomyelin originating from other sources.

0.1070 gm. substance gave 0.2914 gm. CO_2 and 0.1266 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	75.29
H	13.20	12.24